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Award Number: DAMD17-97-2-7002

TITLE: Diagnosis and Dosimetry of Exposure to Sulfur Mustard: Development of Standard Operating Procedures; Further Exploratory Research on Protein Adducts

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REPORT DATE: January 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5400

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | January 2000 | Final (1 Jan 97 - 31 Dec 99) |

**4. TITLE AND SUBTITLE**

Diagnosis and Dosimetry of Exposure to Sulfur Mustard: Development of Standard Operating Procedures; Further Exploratory Research on Protein Adducts

**5. FUNDING NUMBERS**

DAMD17-97-2-7002

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**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

In continuation of previous grants, Standard Operating Procedures (SOPs) have been developed for retrospective detection of exposure to sulfur mustard, *i.e.*, an immunoslotblot assay for DNA adducts in blood and skin, and the modified Edman procedure for determination of adducts to the N-terminal valine in hemoglobin. Both SOPs were validated (day-to-day variability, inter- and intraindividual variation) and could be properly set up and carried out at another institute (MRICD) within one working day. *In vivo* persistence studies showed that in hairless guinea pigs the DNA adduct in blood is still detectable at 14 days after i.v. administration of sulfur mustard (0.5 LD50), while the adduct to N-terminal valine of hemoglobin can still be analyzed after 56 days. In skin the DNA adduct level is only slowly decreasing during the first 3 days and is marginal at 17 days after skin exposure of the hairless guinea pig. In marmosets the DNA adduct is detectable in blood during 7 days after i.v. administration of sulfur mustard (4.1 mg/kg). The adduct to N-terminal valine of hemoglobin is still detectable 94 days after exposure. Furthermore, exploratory research has been performed aiming at the development of a fieldable immunochemical assay for sulfur mustard adducts with hemoglobin, albumin and keratin. Upon exposure of human blood to sulfur mustard, the major adducts in albumin are formed with histidine and cysteine-34. Exposure to ≥ 10 nM sulfur mustard was determined by LC-tandem MS analysis of a tripeptide containing this adducted cysteine in a pronase digest of only 3 mg albumin, *i.e.*, the most sensitive marker for exposure to sulfur mustard developed so far. Exposure of Iranian victims of the Iran-Iraq conflict was detected by using both this procedure and the modified Edman procedure. Treatment at pH 13 released 80% of the bound radioactivity as [$^{14}$C]thiodiglycol from keratin isolated from [$^{14}$C]sulfur mustard exposed human callus, which suggests that most of the adducts formed are esters of glutamic and aspartic acid residues. Exposure of human callus to 10 μM of sulfur mustard could be detected by GC-MS analysis of released thiodiglycol after derivatization. In a similar way, exposure of human skin to saturated sulfur mustard vapor could be detected. Three partial sequences of hemoglobin, the T5 fragment from albumin, and three partial sequences of keratin were synthesized and used as haptens for raising antibodies, which contain adducted histidine, cysteine, and glutamic- or aspartic acid, respectively. Several clones have been obtained. Some of the antibodies directed against keratin adducts showed binding to the horny layer of human skin exposed to sulfur mustard (Ct 1040 mg.min m-3). Such antibodies can directly be applied to human skin, which opens the way for development of an immunochemical kit for field detection of skin exposure.

**14. SUBJECT TERMS**

sulfur mustard, human blood, skin, adducts, DNA, protein, hemoglobin, albumin, keratin, retrospective detection, biomonitoring, immunoslotblot, modified Edman, GC-MS, LC-tandem MS, peptide synthesis, haptens, monoclonal antibodies, immunochemical detection

**15. NUMBER OF PAGES**

143

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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7-04-2000

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N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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PI – Signature Date

7-04-2000

ACKNOWLEDGEMENTS

Many thanks are due for the crucial contributions of Alex Fidder and Mrs. Roos Mars-Groenendijk who were primarily responsible for the organic chemistry and the analytical chemistry, and for the biochemistry and immunochemistry, respectively, described in this report. The authors are grateful to Mrs. Lai Fun Chau and Ms. Saskia de Kant for performing synthetic and analytical experiments, to Mrs. Helma van Dijk-Knijnenburg for development of the immunoslotblot assay, to Albert Hulst, Ad de Jong, Carla Degenhardt, Ben van Baar and Eric Wils who carried out the mass spectrometric analyses, to Simon van Krimpen who did the NMR analyses, and to Cees van der Wulp for his assistance to quantitative immunofluorescence microscopy experiments.
The authors are grateful to Professor Dr. T.J.F. Savelkoul (Academic Hospital Utrecht, The Netherlands) for kindly providing us with blood samples from Iranian victims, to Dr. J.M. van Noort (TNO Prevention and Health, Leiden, The Netherlands) for amino acids analysis of a keratin sample and to Micromass UK Limited (Wythenshawe, U.K.) for performing a number of Q-Tof MS analyses.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES Page

# I INTRODUCTION

The rapid proliferation of chemical weapons, the confirmed use of sulfur mustard, sarin and tabun in the Iran-Iraq conflict (Report of the specialists, 1984), the threat of chemical warfare in the Gulf War and the attack with nerve agents by terrorists in Tokyo and Matsumoto (Croddy, 1995) have stressed the need for reliable methods to detect nature and extent of poisoning with chemical warfare agents.

Within the framework of previous grants (DAMD17-88-Z-8022 and DAMD17-92-V-2005) we have worked on the development of methods for diagnosis and dosimetry of exposure to sulfur mustard (Benschop, 1991; Fidder *et al.*, 1994, 1996a, 1996b; Van der Schans *et al.*, 1994a; Benschop and Van der Schans, 1995; Noort *et al.*, 1996a, 1997; Benschop *et al.*, 1997). Our approach is based upon the development of monoclonal antibodies against adducts of sulfur mustard with DNA and proteins for use in a variety of immunochemical assays and upon the development of procedures for GC-MS and LC-MS-MS analyses to validate the immunochemical assays. The major results obtained were:

1. Several immunochemical assays have been developed with monoclonal antibodies raised against the N7-adduct of sulfur mustard with guanine (N7-HETE-Gua[1]) in DNA, enabling the sensitive detection of N7-HETE-Gua in DNA of human white blood cells and in DNA of human skin after exposure to sulfur mustard.
2. Since it was expected that adducts of sulfur mustard to proteins are more persistent than those to DNA, we initiated also the production of antibodies against sulfur mustard-protein adducts. We obtained monoclonal antibodies against S-HETE-$Cys_{93}$ of the β-chain of human hemoglobin allowing the detection of exposure of human blood to 50 μM sulfur mustard.
3. A modified Edman procedure for selective cleavage of the alkylated N-terminal valine in hemoglobin was developed which detects exposure of human blood to sulfur mustard concentrations as low as 0.1 μM by means of GC-NCI/MS after further derivatization (Fidder *et al.*, 1996a).
4. The modified Edman procedure and the immunoslotblot assay allowed detection of exposure to sulfur mustard of two Iranian victims of the Iran-Iraq war in blood samples taken 22-26 days after exposure from analyses of globin adducts in erythrocytes and DNA adducts in lymphocytes, respectively (Benschop *et al.*, 1997).

In the present work, we have extended these investigations. The following aspects are dealt with:

A. The immunoslotblot assay of sulfur mustard adducts to DNA in human blood and skin and GC-MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin of human blood were developed into standard operating procedures for use in the appropriate environment.

In more detail:

- The various steps involved in the immunochemical assay were simplified and minimized as much as possible for application under field conditions, in analogy with our research on a biological radiation dosimeter.
- The procedure for isolation of globin from human blood, treatment with the modified Edman reagent and further derivatization, and GC-MS analysis of the obtained derivative were optimized with regard to simplicity, sensitivity, and reproducibility.

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[1] HETE: 2-hydroxyethylthioethyl

- The two procedures were used for analysis of blood samples from hairless guinea pigs and marmosets at various time points after i.v. administration of sulfur mustard in order to (i) demonstrate that the two procedures are mutually confirming, (ii) evaluate the dependence of the results on the dose, and (iii) obtain data on the persistence of the various adducts. The immunochemical assay was also validated after application of the agent on the skin of the hairless guinea pig.
- It was demonstrated that the developed procedures can be performed satisfactorily at another institute, *i.e.*, the U.S. Army Medical Research Institute of Chemical Defense.
- Standard operating procedures were drafted for the two assays.

B. Further exploratory research for the development of immunochemical assays of protein adducts was performed based on our present results and evolved insights.

Firstly, these investigations were aimed at enhancing the sensitivity of an immunochemical assay of hemoglobin adducts by means of:

- Further characterization of the available monoclonal antibodies raised against S-HETE-$Cys_{93}$ of the β-chain of human hemoglobin, and use of these antibodies for the development and improvement of immunochemical assays,
- Improvement of the sensitivity of the assay by detecting also adducts to other sites of hemoglobin, particularly to histidine residues, by using a cocktail of monoclonal antibodies in the immunochemical assay.

Secondly, the further exploratory research was directed to adducts with albumin, which is more accessible to the agent in blood than hemoglobin, and to keratin in skin, which gives prospects to detect adducts by way of reagents sprayed on the skin. The research was based on the promising results obtained in our previous studies and on a systematic approach for immunochemical analysis of adducts which has evolved from these results. This approach involves the following steps.

- Semi-quantitative analysis of amino acid-adducts after acidic and protease-catalyzed hydrolysis.
- Determination of sites of alkylation within the tertiary structure of the protein from electrospray LC-MS-MS analyses of adducted peptides in tryptic digests of the alkylated protein.
- Molecular modeling of the adducted protein based on the above-mentioned analyses, giving a lead to the synthesis of the most accessible haptens for generation of monoclonal antibodies against the adducts.
- Synthesis of synthons derived from adducted amino acids which are suitable for solid phase synthesis of peptide haptens and subsequent synthesis of multiple haptens for use in immunization experiments.
- Development of immunochemical assays based on the simultaneous use of several monoclonal antibodies.
- Validation of the immunochemical results by means of GC-MS or LC-MS-MS analysis of the most suitable amino acid-sulfur mustard adduct formed in the sulfur mustard-treated protein.

# II MATERIALS AND INSTRUMENTATION

## II.1 Materials

WARNING: Sulfur mustard is a primary carcinogenic, vesicant, and cytotoxic agent. This compound should be handled only in fume cupboards by experienced personnel.

Technical grade sulfur mustard was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5%. The following compounds were synthesized as described previously (Benschop and Van der Schans, 1995): Nα-Boc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine methyl ester, 2-(2-aminoethylthio)ethanol, Nα-Fmoc-(N1/N3-HETE)histidine and sulfur mustard-$d_8$. The monoclonal antibody 2F8, directed against N7-HETE-guanine (N7-HETE-Gua) in DNA was the same as described previously (Benschop and Van der Schans, 1995).

Thionylchloride was purchased from Janssen Chimica (Tilburg, The Netherlands) and distilled before use. N-methylmorpholin (NMM, Janssen Chimica) was distilled from NaOH at atmospheric pressure before use. N-methylpyrrolidone (NMP, Aldrich Chemie, Bornem, Belgium) was vacuum distilled under a nitrogen atmosphere before use.

9-Fluorenylmethoxycarbonyl (Fmoc) amino acids purchased from Novabiochem (Läufelfingen, Switzerland) were of the L configuration, bearing the following side chain protecting groups: *tert*-butyl (tBu) for aspartic acid, glutamic acid, serine and threonine, trityl for histidine and asparagine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl for arginine, and *tert*-butyloxycarbonyl (Boc) for lysine. Tentagel S AC (Rapp Polymere, Tübingen, Germany) was used as a resin (40-60 mg per peptide, 10 μmol of Fmoc amino acid loading).

The following commercially available products were used:
diethanolamine, gelatin, glycine, poly(ethylene glycol) (PEG 4000), poly(ethylene glycol) (PEG 20,000), proteinase K (Merck, Darmstadt, Germany); acetonitrile (Baker Chemicals, Deventer, The Netherlands); human serum albumin (HSA), pentafluorophenyl isothiocyanate (PFPITC), borane tetrahydrofuran complex solution (1.0 M), sodium ethoxide solution, pentafluorobenzoyl chloride (Fluka, Buchs, Switzerland); N-Boc-1-*tert*-butyl-L-glutamate (Boc-Glu-OtBu), dl-dithiothreitol (DTT), iodoacetic acid sodium salt, TPCK trypsin, α-chymotrypsin, V8 protease, aminopterine, bovine serum albumin, 5-bromodeoxyuridine (BrdU), calf thymus DNA, human hemoglobin, hypoxanthine, RNAse A, pronase Type XIV from Streptomyces Griseus (E.C. 3.4.24.31), tween 20 (polyoxyethylenesorbitan monolaurate) (Sigma Chemical Co., St. Louis, MO, U.S.A.); immobilized TPCK-trypsin (14 units/ml gel), Nonidet-P40 (N-P40), heptafluorobutyrylimidazole (Pierce, Rockford, IL, U.S.A.); 9-Fluorenylmethylchloroformate (Fmoc-Cl), β-mercaptoethanol, L-cysteine, guanidine.HCl, Tris.HCl, EDTA (Janssen, Beerse, Belgium); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), N-Boc-1-*tert*-butyl-L-aspartate (Boc-Glu-OtBu) (Novabiochem); trifluoroacetic acid, thiodiglycol (Aldrich, Brussels, Belgium); fetal calf serum (FCS; LCT Diagnostics BV, Alkmaar, NL); goat-anti-mouse-Ig-alkaline phosphatase, goat anti-mouse-IgG-alkaline phosphatase (KPL, Gaithersburg, USA); microtiter plates (96 wells; polystyrene 'high binding'), microtiter plates (96-wells culture plates) (Costar, Badhoevedorp, The Netherlands); 4-methylumbelliferyl phosphate (MUP), dispase, RNAse T1, protease inhibitor cocktail mini tablets, (Boehringer, Mannheim, Germany); penicillin (Gist Brocades, Delft, The Netherlands); rabbit-anti-mouse-Ig-horse radish peroxidase (Dakopatts, Glostrup, Denmark); FITC-labeled 'goat-anti-mouse' (Southern Biotechnology Associates, Birmingham, AL), RPMI-1640 medium (Gibco BRL, Breda, The Netherlands); skimmed milk powder, less than 1% fat,

(Campina, Eindhoven, The Netherlands); sodium azide (BDH, Poole, UK); streptomycin (Biochemie, Vienna, Austria); heparin (5000 IU/ml, Vitrum, Stockholm, Sweden); racemic ketamine (Vetalar®, Parke Davis, Morris Plains, NJ); Nembutal® (Na-pentabarbital), dormicum/hypnorm (AUV, Cuijk, The Netherlands); and [$^{14}$C]bromoacetic acid (Amersham, Houten, The Netherlands).
Carbosorb and Permablend scintillation cocktail were obtained from Canberra Packard (Tilburg, The Netherlands).

Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10 kDa filters were obtained from Schleicher & Schuell (Dassel, Germany). SepPak Florisil and SepPak C-18 cartridges were obtained from Waters (Bedford, MA).

Human callus was obtained from chiropodists. Human skin resulting from cosmetic surgery was obtained from a local hospital (with consent of the patient and approval of the TNO Medical Ethical Committee). Blood samples from Iranian victims were obtained from the Academic Hospital Utrecht (The Netherlands; see also Subsection IV.2.7)

## II.2 Instrumentation

UV absorbance and UV spectra were recorded on a UV/VIS Spectrometer, Lambda 40 (Perkin Elmer, Breda, The Netherlands).

HPLC was performed using a Gilson (Villers le Bel, France) HPLC system consisting of a 305 master pump, a 306 slave pump, a 805 manometric module and a 811C dynamic mixer, in combination with a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden). The eluent (flow rate: 1 ml/min) was 0.1% trifluoroacetic acid (TFA) in $H_2O$ with a linear gradient to 0.1% TFA in $CH_3CN/H_2O$ (various ratios). The eluate was monitored at 214 or 254 nm with a Spectroflow 757 UV detector (Applied Biosystems, Ramsey, NJ).
Radiometric detection was performed with a Radiometric Flo-one/Beta A-500 radiochromatography detector (Canberra Packard) using Flo-Scint A (Canberra Packard) as a scintillation cocktail.

TLC was performed on Merck HPTLC plates (60F 254; 5x10 cm) or on Merck RP-18 plates (5x20 cm).
FPLC analyses were carried out on a PepRPC 5/5 column using two pumps P-500, a controller LCC-501 plus and a UV-M II monitor (all Pharmacia, Uppsala, Sweden).
Gel filtration on Sephadex G-75 and LH-20 (Pharmacia) was performed with a P-1 pump, GP-250 gradient programmer, Frac-100 fraction collector, a UV-1 optical unit (254 nm) and a UV-1 control unit (Pharmacia).

LC-tandem MS spectra were recorded on a VG Quattro II triple quadrupole mass spectrometer (Micromass, Altrincham, U.K.). The analyses were carried out with multiple reaction monitoring at a dwell time of 2 s, unless stated otherwise. Operating conditions were: capillary voltage 3.6 kV, cone voltage 25 V, collision energy 15 V, gas (argon) cell pressure 0.3 Pa, and source temperature 120 °C. The LC system comprised a reverse phase C18 column (Lichrosorb, 5 µm particles) with water/acetonitrile/formic acid (80/20/0.1, v/v/v) as an eluent. The flow rate was 0.8 ml/min with a split of ca. 1/10 to the mass spectrometer; the injection volume was 10-40 µl. A few analyses were performed with a Q-Tof MS (Micromass, Wythenshawe, U.K.) in the electrospray positive ion mode, using a similar LC system.

GC-NCI/MS analyses were performed with:

- a VG70-250S mass spectrometer (Fisons Instruments, Altrincham, U.K.) operated in the NCI mode (methane) with a source temperature of 200 °C, an ionization energy of 70 eV, and an ion source pressure of 2 mPa. The gas chromatograph (HP 5890A) was equipped with an on-column injector (Carlo Erba, Milan, Italy) and a CPSil 5CB fused silica capillary column (length 50 m, i.d. 0.32 mm, film thickness 0.25 µm; Chrompack, Middelburg, The Netherlands). The oven of the chromatograph was kept at 120 °C for 5 min; the temperature was then programmed at 15 °C/min to 275 °C and subsequently kept at this temperature for 10 min.
- a HP 5973 mass selective detector connected to a HP 6890 GC system with an HP 7673 autoinjector, using pulsed splitless injection. The system was operated in the NCI mode (methane) with a source temperature of 150 °C and an ionization energy of 70 eV. The column used was a CPSil 5 CB fused silica capillary column (length 50 m, i.d. 0.32 mm, film thickness 0.25 µm; Chrompack, Middelburg, The Netherlands) or a Hewlett Packard 19091J-433 HP-5 column (5% phenyl methyl siloxane; length 30 m, i.d. 0.25 mm, film thickness 0.25 µm). The oven of the chromatograph was kept at 120 °C for 5 min, the temperature was then progammed at 15 °C/min to 275 °C and subsequently kept at this temperature for 10 min. The injection volume was 1 µl (containing about 1% of the total sample).

For thermodesorption/cold trap (TCT) injection, the sample (50 µl) was tranferred onto a clean Tenax tube (Chrompack). A helium flow (50 ml/min) was applied during 30 min in order to evaporate the solvent. Next, the Tenax tube was placed in a TCT unit (Chrompack) and rapidly heated to 250 °C while holding the cold trap at - 125 °C. The helium flow rate was 15 ml/min. After 10 min, the helium vent located directly behind the cold trap was closed resulting in a helium flow rate through the analytical column of 1.5 ml/min and the cold trap was flash-heated to 250 °C.

$^{1}H$- and $^{13}C$-NMR spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) VXR 400S spectrometer operating at 400.0 MHz and 100.6 MHz, respectively. Chemical shifts are given in ppm relative to tetramethyl silane. The solvent signals at 2.525 ppm (residual $Me_2SO$-$d_5$ in $Me_2SO$-$d_6$) or 7.260 ppm (residual $CHCl_3$ in $CDCl_3$) served as a reference for $^{1}H$ NMR spectroscopy, whereas the solvent signals at 39.6 ppm ($Me_2SO$-$d_6$) or 77.1 ppm ($CDCl_3$) were used as a reference for $^{13}C$ NMR spectroscopy.

Radioactivity countings were performed on a Packard Tri-Carb series Minaxi (Downers Grove, IL, U.S.A.) or a Packard Mark III liquid scintillation spectrometer with Picofluor 30 (Packard) as a scintillation cocktail.
Thin layer chromatograms of radioactive products were scanned using a Bioscan System 200A Imaging Scanner (Bioscan Inc., Washington, DC, U.S.A.).

Peptides were synthesized on an Abimed (Langenfeld, Germany) AMS 422 peptide synthesizer. Peptides were analyzed by FPLC using a reversed phase PepRPC 5/5 column. Linear gradient elution (1 ml/min) was performed from 0.1% TFA/$H_2O$ to 0.1% TFA/70% $CH_3CN$ in 20 min. Detection was at 214 nm.

SDS-PAGE on albumin was performed on a BioRad system, applying Coomassie brilliant blue R250 coloration.

Microtiter plates were washed using the Skanwasher 300 (Skatron Instruments, Norway; Costar). The fluorescence on microtiter plates (excitation at 355 nm; emission at 480 nm) was recorded with a Cytofluor II (PerSeptive Biosystems, Framingham, MA).

Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 $mm^2$ slots) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). DNA was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, The Netherlands). A Enhanced Chemiluminescence Blotting Detection System (Boehringer) was used for the detection of peroxidase activity. The developed film was scanned with a densitometer (Ultroscan XL, Pharmacia). In later experiments the chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

# III EXPERIMENTAL PROCEDURES

## III.1 Development of an immunochemical assay of sulfur mustard adducts to DNA as a Standard Operating Procedure

### III.1.1 Treatment of DNA with sulfur mustard

A solution of double-stranded calf thymus DNA (1 mg/ml) in TE buffer (10 mM Tris-HCl, 1 mM $Na_2EDTA$, pH 7.4) was treated with sulfur mustard in acetonitrile (0.01-10 µM; final acetonitrile concentration 1%) at 37 °C for 30-60 min and subsequently stored at -20 °C. In alternative experiments, the DNA solution was mixed with sulfur mustard solution at room temperature and subsequently incubated in an incubator at 37 °C or at room temperature.

### III.1.2 Treatment of human blood with sulfur mustard and isolation of blood cells

Venous blood of human volunteers (10 ml, with consent of the donor and approval of the TNO Medical Ethical Committee) was collected in evacuated glass tubes, containing $Na_2EDTA$ (15 mg). The blood sample was treated with sulfur mustard in acetonitrile (0.01-10 µM; final acetonitrile concentration 1%) at 37 °C for 30-60 min. In alternative experiments, blood was mixed with an appropriate sulfur mustard solution at room temperature and subsequently incubated in an incubator at 37 °C or at room temperature.

### III.1.3 DNA isolation from human blood

Several DNA isolation procedures have been applied and are described below. In general, these procedures include the lysis of erythrocytes, the lysis of the white blood cells (white blood cells), sometimes combined with a treatment with proteinase K, an RNAse A and T1 treatment, protein precipitation, DNA precipitation, solvation of the DNA pellet, and measurement of the DNA concentration. The isolation was carried out on 300 µl of whole blood, except in the case of the phenol/chloroform/isoamylalcohol extraction method in which 1 ml of blood was used. In all cases, the methods have been applied to both frozen and fresh blood. After the final wash with 70% ethanol and drying on air or with a 'Speedvac', the DNA was resuspended in TE buffer. The DNA-concentration was determined spectrophotometrically ($\varepsilon_{260nm}$ = 6,600 $l.mol^{-1}.cm^{-1}$, expressed per mol of nucleotide) in a 20-fold dilution of a 4-µl aliquot of the DNA solution, with an uncertainty of about 5% (standard deviation). The purity of the solution was checked by determining the $A_{260}/A_{280}$ ratio of the DNA solution.

*Isolation with phenol/chloroform/isoamylalcohol extraction*

Lysis of the erythrocytes in blood (1 ml) was brought about by incubation of the cell suspension with three volumes of freshly prepared lysis buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, pH 7.4) at 0 °C for 5 min. After centrifugation for 15 min at 400*g* (4 °C), the supernatant, containing the hemoglobin, was removed. The pelleted white blood cells were washed twice with PBS and resuspended in TE buffer (1 ml). Sodium dodecyl sulfate (SDS; final concentration 1%, w/v) was added to lyse the cells and proteinase K (final concentration 500 µg/ml) to digest the protein. The lysates were incubated overnight at 37 °C. DNA was purified by gently shaking with an equal volume of phenol, saturated with 1 M Tris-HCl, pH 8, for 15 min, followed by separation and removal of the phenol layer and two additional extractions with equal volumes of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) and chloroform/isoamylalcohol (24:1, v/v), respectively. After addition of 0.1 volume of 3 M sodium acetate, 1 mM $Na_2EDTA$, pH 5.5, the DNA was precipitated with two volumes of absolute ethanol, pre-cooled at -20 °C. After centrifugation at 3000*g* for 3 min, the pellet was washed in 70% ethanol and dissolved

overnight in TE buffer (1 ml). Next, the solution was incubated with RNAse A (final concentration 75 µg/ml, heated at 80 °C for 5 min to destroy any DNAse activity) and RNAse T1 (final concentration 75 units/ml) at 37 °C for 2 h in order to digest the RNA and subsequently with proteinase K (100 µg/ml) for 1 h to digest protein. The DNA was purified by repeating the phenolic extraction procedure and alcohol precipitation as described above. The DNA was dissolved overnight in TE buffer (300 µl) under continuous vibration at room temperature.

*Isolation by using a PureGene kit (Biozym)*
RBC Lysis Solution (900 µl) was added to blood (300 µl) in order to lyse the red blood cells and the mixture was centrifuged at 14,000*g* for 20 s. The pelleted white blood cells were lysed with Cell Lysis Solution (300 µl). In some cases, proteinase K was added to a final concentration of 100 µg/ml. Lysis was achieved under continuous slow shaking on a rotating wheel at 37 °C until a clear solution was obtained, lasting about 2 h. Then, RNAse A treatment (1.5 µl; 50 µg/ml) was carried out for 15 min at 37 °C, followed by cooling to 20 °C and addition of Protein Precipitation Solution (100 µl). After centrifugation at 14,000*g* for 3 min, the supernatant was transferred to a tube containing isopropanol (300 µl) to precipitate the DNA. After centrifugation (14,000*g* for 3 min), the pellet was washed with 70% ethanol (300 µl), dried in a 'Speedvac' and dissolved overnight in TE buffer (100 µl) under continuous vibration at room temperature. To speed up the procedure the DNA pellet could also be dissolved by incubation at 65 °C for 30 min. However, the latter modification might result in some loss of N7-HETE-Gua from the DNA and lower DNA yields.

*Isolation by using a Xtreme™ Genomic DNA Purification Kit (Pierce)*
Reagent A (900 µl) was added to blood (300 µl) and the mixture was centrifuged (1300*g*, 5 min) after shaking at room temperature during 5 min. The supernatant was discarded, Reagent B (340 µl) was added and the mixture was vortexed briefly to resuspend the pellet and left overnight at room temperature. After addition of a RNAse A solution (2.5 µl; 50 µg/ml), the solution was incubated at 37 °C for 30 min. Then, 5 M Sodium Perchlorate Solution (100 µl) was added and the mixture was shaken at 37 °C for 20 min, followed by 20 min incubation at 65 °C. After cooling to room temperature, the DNA was extracted by adding chloroform (580 µl), shaking for 20 min at room temperature and centrifugation at 1300*g* for 1 min. Then, XtremeTM Silica suspension (45 µl) was added and the mixture was centrifuged at 1300*g* for 4 min. The DNA-containing phase was poured off and ethanol (880 µl) was added to precipitate the DNA. The DNA was centrifuged at 4000*g* for 5 min and washed with 70% ethanol (0.9 ml). The pellet was dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

*Isolation by using a Pharmacia kit*
Equal volumes (300 µl) of whole blood and ice-cold Cell Lysis Buffer were mixed gently and incubated on ice for 5 min and then centrifuged at 4000*g* for 1 min to pellet the nuclei. The supernatant was discarded and the pellet washed two times with Cell Lysis Buffer, 1:1 diluted with water (500 µl), by gently mixing and subsequent centrifugation. To the creamy white pellet, Extraction Buffer (50 µl) was added. The mixture was vortexed gently and left overnight at room temperature. Subsequent to a 10 min incubation period at 55 °C, Application Buffer (800 µl) was added and the mixture was incubated at room temperature for 5 min. Part of the supernatant (ca. 400 µl) was brought on a pre-spun MicroSpin Column and mixed. After 1 min, the column and the support tube were spinned at 735g for 2 min. The remaining half of the supernatant (400 µl) was added to the same pre-spun column and mixed. After 1 min, the column and the support tube were spinned at 735g for 2 min. After washing of the column with Wash Buffer (400 µl), the DNA was eluted from the column by adding twice Elution Buffer (200 µl), followed by centrifugation.

The DNA was precipitated by adding isopropanol (320 µl) to eluted DNA (400 µl) and leaving it for 10 min at room temperature, followed by centrifugation at 735*g* for 10 min. The pellet was washed with 70% ethanol (500 µl), dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

*Isolation by using a Wizard™ Genomic DNA Purification Kit (Promega)*
Whole blood (300 µl) was added to Cell Lysis Solution (900 µl) and gently mixed. After 10 min of incubation at room temperature to lyse the red blood cells, the suspension was centrifuged at 14,000*g* for 1 min. The supernatant was discarded and the pellet resuspended in the remaining supernatant. Then, Nuclei Lysis Solution (300 µl) was added and pipetted 5 times to lyse the white blood cells. The suspension was incubated at 37 °C until a clear solution was obtained (2 h). RNAse Solution (1.5 µl) was added to the nuclear lysate and the misture was incubated at 37 °C for 15 min. After cooling to room temperature, Protein Precipitation Solution (100 µl) was added. The mixture was vortexed vigorously for 20 s and centrifuged at 14,000*g* for 3 min. The supernatant was transferred to a clean tube containing isopropanol (300 µl) at room temperature, mixed gently and centrifuged at 14,000*g* for 1 min. The pellet was washed with 70% ethanol (500 µl), air-dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

*Isolation by using a Stratagene DNA MicroExtraction Kit (Westburg)*
To Solution 1 (1.2 ml) blood (300 µl) was added and incubated on ice for 2 min. The nuclei were pelleted at 14,000*g* for 10 min, washed once with Solution 1 and centrifuged again. The pellet was resuspended in Solution 2 (330 µl) and pronase solution (1 µl; 225 mg/ml) was added. After incubation at 37 °C for 2 h, the mixture was chilled on ice for 10 min. Solution 3 (120 µl) was added to precipitate the protein. After centrifugation at 14,000*g* for 15 min, the supernatant was transferred to another tube, RNAse (1 µl; 10 mg/ml) was added and the mixture was incubated at 37 °C for 15 min. DNA was precipitated by addition of 2 volumes of 100% ethanol, cooled at -20 °C for 10 min and centrifuged at 14,000*g* for 5 min at 4 °C. The precipitate was washed with 70% ethanol, dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

*Isolation by using a DNA Isolation Kit for Mammalian Blood (Boehringer Mannheim)*
To Red Blood Cell Lysis Buffer (900 µ1) whole blood (300 µl) was added. After gently shaking for 10 min, the mixture was centrifuged at 14,000*g* for 20 s. The supernatant was discarded and the pellet was resuspended in the residual supernatant. White Cell Lysis Buffer (300 µl) was added and mixed thoroughly by vortexing. After a clear solution was obtained (45 min at 37 °C), RNAse A (1.5 µl; 50 µg/ml) was added to a final concentration of 0.02 µg/ml and incubated at 37 °C for 15 min. After cooling to room temperature, Protein Precipitation Solution (150 µl) was added. The mixture was vortexed thoroughly and then centrifuged at 12,000*g* for 10 min. The supernatant was poured carefully into another tube and 2 volumes ethanol were added at room temperature, gently mixed and centrifuged at 12,000*g* for 10 min. The pellet was washed with 70% ethanol (1 ml), dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

III.1.4 DNA isolation from human skin

DNA from human skin was isolated by separating the epidermis from the dermis by dispase treatment overnight, lysis of the epidermal layer and further following the same procedure as for blood after lysis of the red blood cells and pelleting the white blood cells (see Subsection III.1.3). To this purpose skin biopsies (about 3 × 3 mm) were first treated overnight at 4 °C in a 3-cm petri-dish with the enzyme dispase (2.4 mg/ml PBS; 3 ml) to separate the epidermis from the dermis (by

layering the pieces of skin on the dispase solution and shortly emersing the pieces in it). The epidermis was transferred to an Eppendorf tube and then the lysis solution was added, followed by RNAse A (3 µl of 75 µg/ml) treatment at 37 °C for 1 h. The procedure was continued as described for isolation of DNA from the blood samples by using a PureGene kit of Biozym.

III.1.5 DNA denaturation

Double-stranded calf thymus DNA or DNA from sulfur mustard-exposed human white blood cells were made single-stranded by thermal denaturation in TE buffer containing 4.1% formamide and 0.1% formaldehyde (50 µg DNA/ml) at 52 °C for 15 min, followed by rapid cooling on ice and storage at -20 °C.
In later experiments, thermal denaturation was carried out in 10-fold diluted TE buffer (0.1TE) instead of TE buffer. It appeared to be essential that, after denaturation, the DNA samples have been frozen at least one time before application in the immunoslotblot assay (see Subsection IV.1.3).

III.1.6 Immunoslotblot procedure for N7-HETE-Gua

Several modifications were applied to the previously described procedure (Benschop and Van der Schans, 1995). So far, the following procedure appeared to be the optimum method. In the immunoslotblot assay the single-stranded DNA containing N7-HETE-Gua was first slotblotted onto a nitrocellulose filter. Thermally denatured DNA was diluted in PBS to a final concentration of 5 µg/ml. The solution (200 µl) was spotted on a nitrocellulose filter. Ten positions on the 96-blots filter were occupied by calibration samples of DNA with adduct levels in the range of 0-10 N7-HETE-Gua/$10^7$ nucleotides. All samples were blotted in duplicate on the same filter. After blotting, the slots were rinsed with PBS. The filters were dried on air and the DNA was immobilized by UV crosslinking (50 mJ/$cm^2$). The next steps in the procedure, *i.e.*, treatment with blocking solution, 1st antibody (2F8, directed against N7-HETE-Gua in DNA) and 2nd antibody (rabbit-anti-mouse-Ig-horse radish peroxidase), were the same as described previously (Benschop and Van der Schans, 1995). The solutions A and B of the chemiluminescence blotting detection system were mixed (100:1) and equilibrated for 1 h at 25 °C before addition to the filter. The filters were incubated for 1 min in substrate and then placed in a plastic bag. Excess of liquid was pressed out. Next, the filters were placed in a luminometer and the chemiluminescence was measured.
A blood or skin sample not exposed to sulfur mustard and a number of sulfur mustard treated calf thymus DNA samples were always processed simultaneously in the immunoslotblot assay and applied on the same nitrocellulose filter, as blank and calibration samples, respectively.

III.1.7 Standard Operating Procedure for immunoslotblot assay of sulfur mustard adducts to DNA in blood and skin

As a result of the applied modifications and improvements, the following SOP for the immunoslotblot assay has been drafted.

*Materials*

Collection of blood samples
10-ml glass EDTA vacutubes
freezer, -20 °C;

| | |
|---|---|
| **Collection of skin/blister samples** | |
| Capped Eppendorf tubes (1.5 ml) | |
| Freezer, -20 °C | |
| **DNA isolation** | |
| DNA isolation kit | PureGene kit, Biozym |
| Dispase | Boehringer Mannheim, Germany |
| PBS (sterile) | Phosphate Buffered Saline (Dulbecco's) |
| Capped Eppendorf tubes (1.5 ml) | |
| Gilson pipets (20, 200, 1000 µl) and tips | |
| Eppendorf centrifuge, model 5417 C | |
| Proteinase K | |
| Rnase A | |
| Isopropanol | |
| 70% Ethanol | |
| Filterpaper | |
| Water bath at 37 °C | |
| Rotating wheel shaker | |
| Incubator at 37 °C | |
| Vibrator | Titertek (Flow) |
| 0.1TE buffer | 1 mM Tris-HCl, 0.1 mM $Na_2EDTA$, pH 7.4 |
| UV/VIS spectrometer | *E.g.*, Lambda 40, Perkin Elmer, Breda, The Netherlands |
| **DNA denaturation** | |
| Calf thymus DNA calibration samples | Calf thymus DNA exposed to 0, 2.5, 5, 10 and 20 nM sulfur mustard |
| TE buffer | 10 mM Tris-HCl, 0.01 mM $Na_2EDTA$, pH 7.4 |
| MQ water | Purified tapwater, classification I, ISO 3696 |
| Formamide | 99% |
| Formaldehyde | 36.5% |
| 52 °C water bath | |
| Gilsonpipets (20, 200, 1000 µl) and tips | |
| **Immunoslotblot assay** | |
| Nitro cellulose | Protran BA 79, nitrocellulose transfer membrane, 0.1 µm, Schleicher and Schuell |
| Blotting paper | Gel-blotting-paper GB 002 (0.8 mm), Schleicher and Schuell |
| Blotting device | Minifold 1 Dotblot manifold, Schleicher and Schuell |
| Vacuum pump | |
| Glass vacuum flask | |
| 12-channel pipet and tips | |
| Gilsonpipets (20, 200, 1000 µl) and tips | |
| Flat forceps | *e.g.* Millipore |
| Gloves | |
| PBS (sterile) | Phosphate Buffered Saline (Dulbecco's) |
| MQ water | purified tapwater, classification I, ISO 3696 |
| Milkpowder | ELK, skimmed milkpowder, less than 1% fat, Campina, Eindhoven, The Netherlands |

| | |
|---|---|
| 1st Antibody, 2F8 | Directed against against N7-HETE-Gua, culture supernatant, TNO-PML, Rijswijk, The Netherlands |
| 2nd Antibody | Rabbit-anti-mouse-Ig-horse radish peroxidase, Dakopatts, Glostrup, Denmark |
| Enhanced Chemiluminescence Blotting Detection System | Solution A and B, Boehringer Mannheim, Germany |
| Incubation boxes | |
| UV-gene cross-linker | *E.g.*, GS Gene Linker UV chamber, Bio-Rad Laboratories, The Netherlands |
| Waterbath at 25 °C | |
| Luminometer | *E.g.*, MicroBeta Trilux 6 detector system, Wallac, EG & G Berthold |
| Plastic sheets | To pack blots |
| Shaking plate | |
| Stopwatch | |
| Filter paper | |

*Procedure for immunoslotblot asssay of sulfur mustard adducts to DNA in blood*

Sampling

Collect a blood sample (1-10 ml) in an EDTA vacutube from an alleged sulfur mustard victim, mix thoroughly and freeze at –20 °C for storage or transportation (on dry ice).

Isolation of DNA

1. After thawing transfer blood (300 µl) to an 1.5-ml Eppendorf tube.
2. Add RBC lysis solution (900 µl), mix on a rotating wheel, and after 5 min centrifuge at 3,500 rpm (1,300*g*) for 10 min.
3. Lyse the pelleted white blood cells with Cell Lysis Solution (300 µl) supplemented with Proteinase K (100 µg/ml) under continuous shaking on a rotating wheel at 37 °C until a clear solution is obtained, which takes ca. 1 h.
4. Treat with RNase A (1.5 µl, 50 µg/ml) for 15 min at 37 °C, followed by cooling to 20 °C.
5. Add Protein Precipitation Solution (125 µl), mix on a high speed vortex (20 s), and centrifuge at 14,000*g* for 10 min.
6. Transfer the supernatant to a tube containing isopropanol (300 µl) in order to precipitate the DNA, and centrifuge at 7,000 rpm (5,200*g*) for 5 min.
7. Wash the pellet with 70% ethanol (300 µl), centrifuge (7,000 rpm, 5 min), and dry on air for ca. 15 min.
8. Dissolve the pellet in 0.1TE buffer (50 or 100 µl depending on the size of the pellet) under continuous vibration overnight at room temperature. Dissolution can be accomplished within 1 to 2 h when performed with fresh blood.
9. Determine DNA concentration by diluting the DNA solution (4 µl) 20-fold with 0.1TE buffer and measure $A_{260}$ in a 1-cm quartz microcuvette in a UV/VIS spectrometer (1000 × $A_{260}$ = DNA concentration in µg/ml of the undiluted solution). Measure also $A_{280}$ as indication for the purity of the DNA solution. (The $A_{260}/A_{280}$ ratio should be between 1.6 – 1.9.)

DNA denaturation

1. Make up solutions (100 µl) with final concentrations of DNA (50 µg/ml), formamide (4.1%), and formaldehyde (0.1%) in 0.1TE buffer, incubate at 52 °C for 15 min, and cool

rapidly on ice. Store at –20 °C (freezing of the samples at least once is essential). Treat the calf thymus DNA calibration samples in the same way.

Immunoslotblot procedure

1. Dilute the denatured DNA samples in PBS to a final concentration of 5 µg/ml (including the calf thymus DNA calibration samples)
2. Assemble the blotting manifold: connect with vacuum flask and place 2 pieces of blotting paper (wear gloves); make a nitrocellulose filter, cut in a 96-well format, wet (with water and PBS) and place it on the upper part of the manifold (without air bubbles); place the upper part on the other parts and fix the clamps. Switch on the vacuum pump.
3. Spot the DNA solution (200 µl) in duplicate. Do not use position A12 and H1. (These positions are needed as markers for the positioning of the filter in the luminometer cassette.)
4. Wash each dotted sample with PBS (400 µl) by suction through the filter.
5. Take the nitrocellulose filter from the blotting manifold and dry on air for 10-15 min.
6. Cross-link the DNA to the filter by means of illumination with the UV-gene-cross-linker (50 $mJ/cm^2$).
7. Incubate the filter with blocking solution (about 50 ml, 5% milkpowder in PBS + 0.1% Tween 20) at room temperature for 30 min.
8. Wash three times with PBS + 0.1% Tween 20.
9. Incubate the filter with 1st antibody diluted 500-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, overnight at 4 °C under continuous shaking.
10. Wash 4 times with PBS + 0.1% Tween 20; the last three times for at least 15 min each.
11. Incubate the filter with 2nd antibody diluted 1000-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, for 2 h at room temperature under continuous shaking.
12. Wash 4 times with PBS + 0.1% Tween 20; the last three times for at least 15 min each.
13. Incubate solution A (of the Enhanced Chemiluminescence Blotting Detection System) in a waterbath at 25 °C until temperature equilibrium is reached. Mix solution B with solution A in a ratio 1:100 and preincubate the substrate solution for at least 30 min at 25 °C.
14. Remove free (wash) solution from the filter with filter paper, mark position A12 and H1 with ball point (not a felt pen!).
15. Place the filter in a closely fitting box, add 10 ml of substrate solution and incubate for 1 min.
16. Wrap the filter, straight from the substrate in plastic, without air bubbles. Press out liquid, transfer the filter in plastic into the luminometer cassette and place it in the luminometer.
17. Measure luminescence according to the required program. Collect data in a file and calculate for each sample the level of N7-HETE-Gua/$10^7$ nucleotides (in an Excel worksheet).

*Procedure for immunoslotblot asssay of sulfur mustard adducts to DNA in skin*

Sampling

Take skin biopsy (3 × 3 mm) and samples of blisters from possibly exposed sites on the body and freeze at –20 °C for storage or transportation (on dry ice).

Isolation of DNA

1. Treat the skin biopsy overnight at 4 °C in a 3-cm petri-dish with the enzyme dispase (2.4 mg/ml PBS; 3 ml) by layering the pieces of skin on the dispase solution and shortly emersing the pieces in it. Transfer the epidermis to an Eppendorf tube.

2. Lyse the epidermal samples with Cell Lysis Solution (300 µl) supplemented with Proteinase K (100 µg/ml) under continuous shaking on a rotating wheel at 37 °C until a clear solution is obtained, which takes up to about 15 h.

Subsequently, follow steps 4 – 9 of the procedure for isolation of DNA from blood.

DNA denaturation and immunoslotblot procedure
The procedures are identical to the procedures described for sulfur mustard adducts to DNA in blood.

III.1.8 Shortened Standard Operating Procedure for immunoslotblot assay of sulfur mustard adducts to DNA in blood

The original SOP was modified in several steps in order to speed up the procedure. This shortened procedure (with 12 samples) can be carried out within 9 h, but results in ca. 10-fold lower chemiluminescence.

Sampling
1. Collect a blood sample (1-10 ml) in an EDTA vacutube from an alleged sulfur mustard victim, mix thoroughly and freeze at –20 °C for storage or transportation (on dry ice).

DNA isolation
1. Transfer blood (300 µl) after thawing to an 1.5-ml Eppendorf tube.
2. Add RBC lysis solution (900 µl), mix on a rotating wheel, and after 5 min centrifuge at 14,000 rpm for 1 min.
3. Lyse the pelleted white blood cells with Cell Lysis Solution (300 µl) supplemented with Proteinase K (100 µg/ml) under continuous shaking on a rotating wheel at 37 °C for 30 min.
4. Add Protein Precipitation Solution (125 µl), mix on a high speed vortex (20 s), and centrifuge at 14,000*g* for 3 min.
5. Transfer the supernatant to a tube containing isopropanol (300 µl) in order to precipitate the DNA, and centrifuge at 14,000 rpm for 1 min.
6. Wash the pellet with 70% ethanol (300 µl), centrifuge (14,000 rpm, 1 min), and dry on air for about 15 min.
7. Dissolve the pellet in 0.1TE buffer (50 µl), either by continuous vibration (30 min at room temperature) and short vortexing thereafter, or by vortexing shortly, heating at 65 °C for 5 min and vortexing again (if the DNA pellet is not completely solved). Centrifuge at 14,000 rpm for 1 min. Collect the supernantant.
8. Determine concentration of DNA by diluting the DNA solution (4 µl) 20-fold with 0.1TE buffer and measure $A_{260}$ in a 1-cm quartz microcuvette in a UV/VIS spectrometer (1000 × $A_{260}$ = DNA concentration in µg/ml of the undiluted solution). Measure also $A_{280}$ as an indication for the purity of the DNA solution. (The $A_{260}/A_{280}$ ratio should be between 1.6 – 1.9. The DNA concentration should be between 90-200 µg/ml).

DNA denaturation
1. Make up solutions (100 µl) with final concentrations of DNA (50 µg/ml), formamide (4.1%), and formaldehyde (0.1%) in 0.1TE buffer, incubate at 52 °C for 15 min, and cool rapidly on ice. Store at –20 °C for about 30 min (freezing the samples at least once is essential). Treat the calf thymus DNA calibration samples in the same way.

Immunoslotblot procedure

1. Dilute the denatured DNA samples in PBS to a final concentration of 5 µg/ml (including the calf thymus DNA calibration samples)
2. Assemble the blotting manifold: connect with vacuum flask and place 3 pieces of blotting paper (wear gloves); make a nitrocellulose filter, cut in a 96-well format, wet (with water and PBS) and place it on the upper part of the manifold (without air bubbles); place the upper part on the other parts and fix the clamps. Switch on the vacuum pump.
3. Spot the DNA solution (200 µl) in duplicate. Do not use position A12 and H1. (These positions are needed as markers for the positioning of the filter in the luminometer cassette.)
4. Wash each dotted sample with PBS (400 µl) by suction through the filter.
5. Take the nitrocellulose filter from the blotting manifold and dry on air for 10-15 min.
6. Cross-link the DNA to the filter by means of illumination with the UV-gene-cross-linker (50 $mJ/cm^2$).
7. Incubate the filter with blocking solution (about 50 ml, 5% milkpowder in PBS + 0.1% Tween 20) at room temperature for 30 min.
8. Wash three times with PBS + 0.1% Tween 20.
9. Incubate the filter with 1st antibody diluted 500-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, in a 37 °C incubator for 30 min under continuous vibration.
10. Wash 4 times with PBS + 0.1% Tween 20; the last three times for at least 5 min each.
11. Incubate the filter with 2nd antibody diluted 1000-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, in a 37 °C incubator for 30 min under continuous vibration.
12. Wash 4 times with PBS + 0.1% Tween 20 (the last three times for at least 5 min each).
13. Incubate solution A (of the Enhanced Chemiluminescence Blotting Detection System) in a waterbath at 25 °C-until temperature equilibrium is reached. Mix solution B with solution A in a ratio 1:100 and preincubate the substrate solution for at least 30 min at 25 °C.
14. Remove free (wash) solution from the filter with filter paper, mark position A12 and H1 with ball point (not a felt pen!).
15. Place the filter in a closely fitting box, add 10 ml of substrate solution and incubate for 1 min.
16. Wrap the filter, straight from the substrate in plastic, without air bubbles. Press out liquid, transfer the filter in plastic into the luminometer cassette and place it in the luminometer.
17. Measure luminescence according to the required program. Collect data in a file and calculate for each sample the level of N7-HETE-Gua/$10^7$ nucleotides (in an Excel worksheet).

### III.1.9 Day-to-day variability of the immunoslotblot assay for N7-HETE-Gua in DNA in a single blood sample.

The day-to-day variability has been determined as follows. Human blood was exposed to 0, 0.5, 1 or 5 µM sulfur mustard. Blood samples were subsequently frozen in 30-µl aliquots. On 6 different days, samples were thawed, DNA was isolated from the samples, denatured and analyzed in duplicate for the amount of N7-HETE-Gua according to the SOP described in Subsection III.1.7.

### III.1.10 Sensitivity of the shortened Standard Operating Procedure for the immunoslotblot assay of sulfur mustard adducts to DNA in blood

The shortened SOP for the immunoslotblot assay, described in Subsection III.1.8 was applied to blood samples of two donors which had been exposed *in vitro* to sulfur mustard (0, 1 and 5 μM) for 1 h at 37 °C. DNA isolated from the samples was dissolved both at room temperature and at 65 °C.

## III.2 Development of a GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin as a Standard Operating Procedure

### III.2.1 Synthesis of [$^{14}$C]sulfur mustard

To a solution of [$^{14}$C]bromoacetic acid (spec. act. 52 mCi/mmol; 0.5 mmol) in THF was added borane tetrahydrofuran complex solution (0.75 mmol; 1.0 M). The reaction was performed in a 4 ml vial under cooling in an ice bath. After 16 h at room temperature the reaction mixture was quenched by the addition of $H_2O$ (15 μl) and added to a mixture of ethanol (0.5 ml), β-mercaptoethanol (42 μl) and NaOEt solution (21% in ethanol; 225 μl). After 45 min, 2.25 h and 3.75 h, an extra portion (50 μl) of NaOEt solution was added. The mixture was stored overnight at -20 °C. TLC analysis (8% methanol in $CH_2Cl_2$) with radiometric detection showed the presence of radioactive material that coeluted with thiodiglycol. Acetic acid (20 μl) was added and the mixture was concentrated under reduced pressure. The crude [$^{14}$C]thiodiglycol was purified by means of silica gel column chromatography. Elution was performed with a gradient of 0-5% methanol in $CH_2Cl_2$ (50 ml). Fractions were checked with TLC (eluent 6% methanol in $CH_2Cl_2$), by means of detection with $I_2$ vapor and scanning for radioactivity. The main impurity (the disulfide of β-mercaptoethanol), having a slightly larger $R_f$ value, did not contain radioactivity. The fractions containing pure [$^{14}$C]thiodiglycol were collected, concentrated and coevaporated with $CHCl_3$ for removal of methanol. Fractions containing [$^{14}$C]thiodiglycol contaminated with the disulfide were also collected, concentrated and re-chromatographed. The total yield of [$^{14}$C]thiodiglycol was determined with liquid scintillation counting: 14 mCi, spec. act. 52 mCi/mmol (0.27 mmol, 54%).
The purified [$^{14}$C]thiodiglycol was dissolved in $CHCl_3$ (2 ml) and thionyl chloride (96.4 mg; 0.81 mmol; 60 μmol) was added under cooling in an ice bath. The mixture was heated under reflux for 3 h. GC-analysis showed, in addition to the presence of a peak with the same retention time as sulfur mustard, an impurity with a longer retention time. The crude product was diluted with cold sulfur mustard (0.2 mmol; 32 mg) and fractionated by distillation under reduced pressure, as described earlier (Benschop and Van der Schans, 1995). The fractions were analyzed by gas chromatography. Peaks were collected in Carbosorb and radioactivity was determined after addition of Permablend scintillation cocktail. The purity was established from TLC analysis. Two batches of pure [$^{14}$C]sulfur mustard resulted:
batch 1: 23 mg, spec. act. 15 mCi/mmol, radiochemical purity 99+%
batch 2: 9 mg, spec. act. 15 mCi/mmol, radiochemical purity 99+%
One of the impure fractions was combined with an older batch of impure [$^{14}$C]sulfur mustard and redistilled:
batch 3: 18 mg, spec. act. 14 mCi/mmol, radiochemical purity 97%

### III.2.2 Synthesis of [$^{14}$C]sulfur mustard, improved procedure

2-Bromo[1-$^{14}$C]ethanol. To a cooled (0 °C) and stirred solution of [$^{14}$C]bromoacetic acid (ca. 100 mCi; spec. act. 57 mCi/mmol) in THF (1.5 ml) was added borane tetrahydrofuran complex

solution (2.6 ml, 1 M) in the course of 30 min. Next, the ice bath was removed and the reaction mixture was stirred overnight at room temperature. The reaction was quenched with water (0.5 ml) and potassium carbonate (500 mg) was added. The organic layer was removed, while the aqueous layer was washed with diethyl ether (5 × 1 ml). The organic layers were collected, dried with $MgSO_4$, filtrated and concentrated to a small volume under normal pressure using a short distillation bridge, affording 2-bromo[1-$^{14}$C]ethanol as a colorless liquid.

[$^{14}$C]Thiodiglycol. A mixture of ethanol (0.5 ml), sodium ethylate (2.1 mmol, 0.79 ml, 21% solution in ethanol) and 2-mercaptoethanol (147 µl, 2.1 mmol) was added to the obtained 2-bromo[1-$^{14}$C]ethanol and the solution was stirred for 2 h at 50 °C. TLC analysis (8% methanol in $CH_2Cl_2$) indicated almost complete disappearance of 2-mercaptoethanol and the appearance of [$^{14}$C]thiodiglycol, as was concluded from coelution with cold thiodiglycol. A small amount of the non-radioactive disulfide was also visible, having a slightly larger $R_f$ value than [$^{14}$C]thiodiglycol. Ethanol was evaporated under normal pressure and the residue was chromatographed on silica gel applying a gradient from 0 to 5% methanol in $CH_2Cl_2$ in steps of 0.5% (100 ml each) in order to remove the disulfide. Fractions containing pure [$^{14}$C]thiodiglycol (TLC; detection with $I_2$ vapor) were combined and concentrated under normal pressure. Fractions contaminated with the disulfide were re-chromatographed.

Radiochemical yield: 79 mCi (79%, based on 100 mCi of [$^{14}$C]bromoacetic acid). The chemical yield was not determined since the solvent had not been removed completely. TLC analysis (8% methanol in $CH_2Cl_2$) with radiometric detection showed one radioactive compound. Detection with $I_2$ colorization showed one spot.

[$^{14}$C]Sulfur mustard. To [$^{14}$C]thiodiglycol (39.5 mCi) in a 4 ml vial was added concentrated hydrochloric acid (0.5 ml). The vial was sealed with a screw cap and left at 60 °C for 2 h. A two layer system had formed. Water (0.5 ml) and $CH_2Cl_2$ (1 ml) were added to the cooled vial. After thorough mixing, the two layers were separated by centrifugation. The organic layer was collected and the water layer was washed with $CH_2Cl_2$ (3 × 0.5 ml). Next, the combined $CH_2Cl_2$ layers were washed with water (2 × 0.5 ml) and dried over $MgSO_4$. GC analysis showed, in addition to the solvent peak, a single peak which coincided with cold sulfur mustard. After removal of $CH_2Cl_2$ by evaporation under normal pressure, [$^{14}$C]sulfur mustard was obtained in 70% yield (79 mg, 28 mCi) (56% starting from [$^{14}$C]bromoacetic acid). The radiochemical purity was checked by trapping the carbon dioxide evolved from the GC in Carbosorb (3 ml) in 90-s fractions. After addition of Hionic-Fluor LSC cocktail (17 ml), radioactivity was determined by liquid scintillation counting. The main activity (99%) was found in the fraction corresponding with the [$^{14}$C]sulfur mustard peak. The chemical purity, determined with GC, was 99%; spec. act. 56.4 mCi/mmol.

### III.2.3 Incubation of human blood with sulfur mustard, [$^{14}$C]sulfur mustard or sulfur mustard-$d_8$

A 1 M solution of sulfur mustard, [$^{14}$C]sulfur mustard (sp. act. 15 mCi/mmol) or sulfur mustard-$d_8$ in $CH_3CN$ was prepared. For an exposure level of 10 mM, 50 µl of this solution or of an appropriate dilution in $CH_3CN$ was added to human blood (5 ml). After incubation at 37 °C, plasma and erythrocytes were separated by centrifugation at 3,000 rpm.

### III.2.4 Isolation of globin from human blood

Globin was isolated from human blood samples according to Bailey *et al.* (1987). The red blood cells were washed four times with saline and lysed with water. After 30 min in ice/water, they were centrifuged for 30 min at 25,000*g* (4 °C). The supernatant was poured into a stirred mixture of concentrated HCl/acetone (1/100, v/v) at -20 °C. After decanting the supernatant, the precipitate was washed with concentrated HCl/acetone (1/100, v/v), acetone and ether, and

dried. For some experiments, the crude globin was purified via a G-25 Sephadex column, using 0.1 M formic acid, 6 M urea and 50 mM dithiothreithol as an eluent. UV-positive fractions were pooled and dialyzed three times against a 1 mM phosphate buffer, pH 7. Finally, the globin was dialyzed against water for 2 h and lyophilized to give a white fluffy compound.

III.2.5 Original procedure for modified Edman degradation of globin

Globin (20 mg, originating from human or guinea pig blood exposed to sulfur mustard) was dissolved in formamide (2 ml). Pyridine (6 µl) and PFPITC (6 µl) were added. The mixture was incubated overnight at room temperature followed by 2 h at 45 °C. The formamide layer was extracted with diethyl ether (3 × 1.5 ml). The combined ether fractions were dried under a stream of nitrogen and the resulting residue was dissolved in toluene (1 ml). The toluene solution was washed, dried, and concentrated to a small volume (500 µl). Heptafluorobutyrylimidazole (10 µl) was added and the mixture was heated for 10 min at 45 °C. After washing with water (4 × 0.5 ml), the organic layer was dried ($MgSO_4$) and concentrated. The residue was dissolved in 100 µl toluene and analyzed with GC-MS.

III.2.6 Simplified procedure for modified Edman degradation of globin

A mixture of globin (20 mg), PFPITC (6 µl) and pyridine (6 µl) in formamide (2 ml) was heated for 2 h at 60 °C. Subsequently, the mixture was extracted with toluene (3 × 0.5 ml). Separation of the toluene/formamide layers was achieved by freezing in liquid nitrogen. The toluene layers were washed consecutively with water (2 × 0.5 ml), aqueous $Na_2CO_3$ (0.1 M, 0.5 ml) and water (0.5 ml) and concentrated to a small volume (500 µl). Heptafluorobutyryl-imidazole (10 µl) was added and the mixture was heated for 10 min at 45 °C. After washing with water (4 × 0.5 ml), the organic layer was dried ($MgSO_4$) and concentrated. The residue was dissolved in toluene (100 µl) and analyzed with GC-MS.

III.2.7 Analysis of Iranian blood samples for the presence of the sulfur mustard adduct to the N-terminal valine of hemoglobin

Blood samples taken from nine Iranian patients, supposedly exposed to sulfur mustard, were subjected to modified Edman degradation after storage in a freezer for 12 years. Globin was directly isolated from the blood samples using the acid acetone precipitation procedure, see Subsection III.2.4. The globin samples were dried at ambient temperature. Next, the modified Edman degradation was performed (20-50 mg scale; original procedure, see Subsection III.2.5) and after derivatization with heptafluorobutyrylimidazole, the samples were analyzed with GC-MS (SIM, NCI).

III.2.8 Standard Operating Procedure for GC-NCI/MS determination of the sulfur mustard adduct to N-terminal valine in hemoglobin

Globin (20 mg) isolated from blood exposed to sulfur mustard was mixed with globin (20 mg) isolated from blood exposed to sulfur mustard-$d_8$ (10 µM) and dissolved in formamide (2 ml). Next, pyridin (8 µl) and pentafluorophenyl isothiocyanate (8 µl) were added and the mixture was incubated at 60 °C in a heating block for 2 h. After cooling to room temperature, the mixture was extracted with toluene (3 × 1 ml) by means of mixing the toluene with the formamide solution using a Vortex (30 s) and centrifuging in a Jouan RC 10.10 centrifugal evaporator for 2 min (1200 rpm). Next, the samples were frozen in liquid nitrogen in order to achieve a better separation of the two layers. The toluene layers were combined, washed with water (2 × 0.5 ml), aqueous $Na_2CO_3$ (0.1 M, 0.5 ml) and water (0.5 ml). The organic layer was

dried ($MgSO_4$), evaporated to dryness using the centrifugal evaporator and dissolved in toluene (100 µl).
Next, a Florisil cartridge was conditioned with methanol/$CH_2Cl_2$ (1/9, v/v; 2 ml) and $CH_2Cl_2$ (2 ml), respectively. The toluene solution was applied on the cartridge, which was subsequently washed with $CH_2Cl_2$ (2 ml) and methanol/$CH_2Cl_2$ (1/9, v/v; 1 ml). The thiohydantoin was eluted with methanol/$CH_2Cl_2$ (1/9, v/v; 1.5 ml). The latter eluate was evaporated to dryness and dissolved in toluene (100 µl). To this solution, heptafluorobutyryl imidazole (10 µl) was added and the mixture was heated at 60 °C for 30 min. After cooling, the reaction mixture was washed with water (2 × 100 µl), aqueous $Na_2CO_3$ (0.1 M, 100 µl) and finally with water (100 µl). The toluene layer was dried ($MgSO_4$), concentrated to 30 µl and analyzed with GC-MS.
GC-NCI/MS analyses were performed with:
- a VG70-250S mass spectrometer (Fisons Instruments, Altrincham, U.K.) operated in the NCI mode (methane) with a source temperature of 200 °C, an ionization energy of 70 eV, and an ion source pressure of 2 mPa. The gas chromatograph (HP 5890A) was equipped with an on-column injector (Carlo Erba, Milan, Italy) and a CPSil 5CB fused silica capillary column (length 50 m, i.d. 0.32 mm, film thickness 0.25 µm; Chrompack, Middelburg, The Netherlands). The oven of the chromatograph was kept at 120 °C for 5 min; the temperature was then programmed at 15 °C/min to 275 °C and subsequently kept at this temperature for 10 min.
- a HP 5973 mass selective detector connected to a HP 6890 GC system with an HP 7673 autoinjector, using pulsed splitless injection. The system was operated in the NCI mode (methane) with a source temperature of 150 °C and an ionization energy of 70 eV. The column used was a CPSil 5 CB fused silica capillary column (length 50 m, i.d. 0.32 mm, film thickness 0.25 µm; Chrompack, Middelburg, The Netherlands) or a Hewlett Packard 19091J-433 HP-5 column (5% phenyl methyl siloxane; length 30 m, i.d. 0.25 mm, film thickness 0.25 µm). The oven of the chromatograph was kept at 120 °C for 5 min, the temperature was then progammed at 15 °C/min to 275 °C and subsequently kept at this temperature for 10 min. The injection volume was 1 µl (containing about 1% of the total sample).
Ion chromatograms were recorded after monitoring for *m/z* 564 ($M^{-\cdot}$ - 3 HF, analyte) and 572 ($M^{-\cdot}$ - 3 HF, internal standard).

### III.2.9 Day-to-day variability in adduct levels using GC-MS analysis of N-alkylated terminal valine in hemoglobin

The day-to-day variability in adduct levels of a single blood sample exposed to sulfur mustard was determined by using GC-MS analysis of N-alkylated terminal valine in hemoglobin. For this purpose, blood (10 ml) was incubated with a solution of sulfur mustard in acetonitrile (100 µl, 0.5 mM) resulting in a 5 µM exposure level. A blank sample was prepared by incubation of blood (10 ml) with neat acetonitrile (100 µl). After incubation for 2 h at 37 °C, the samples were divided into ten portions of 1 ml and centrifuged (15 min at 400*g*; 4° C). The serum was discarded. No further washing of the erythrocytes was performed. The samples were stored at – 20 °C. At various time points (0, 7, 8, 21, 22, 42, 43, 56, 63, 84 days), globin was isolated and the modified Edman degradation was performed, followed by derivatization with heptafluorobutyryl imidazole, according to the SOP (see Subsection III.2.8). Globin isolated from blood exposed to 10 µM sulfur mustard-$d_8$ was used as an internal standard. The experiment was carried out in duplicate, including blank samples.

## III.3 Validation of the two Standard Operating Procedures

### III.3.1 Intra-individual variation in adduct levels using the immunoslotblot assay for N7-HETE-Gua in DNA

The intra-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard was determined in blood samples taken from 5 donors at 3 time points by using the immunoslotblot assay. Blood samples were collected on different days and incubated with sulfur mustard in acetonitrile (0, 0.5, 1 and 5 µM; final concentration acetonitrile 1%) for 1 h at 37 °C. After exposure, blood samples were stored in 30 µl aliquots at -20 °C and analyzed simultaneously in the immunoslotblot assay.

### III.3.2 Intra-individual variation in adduct levels using GC-MS analysis of N-alkylated terminal valine in hemoglobin

The intra-individual variation of *in vitro* sensitivity of human blood to sulfur mustard was determined in blood samples taken from 5 individuals at 3 time points, by using the GC-MS analysis of N-alkylated terminal valine in hemoglobin. For this purpose, the blood samples (2 ml) were incubated with a solution of sulfur mustard in acetonitrile (500 µM; 20 µl) resulting in an exposure level of 5 µM. A blank sample was prepared by incubation of human blood (2 ml) with neat acetonitrile (20 µl). After incubation for 2 h at 37 °C, globin was isolated and the modified Edman degradation was performed (in duplicate), followed by derivatization with heptafluorobutyryl imidazole, according to the SOP (see Subsection III.2.8), using the VG 70-250S GC-MS system. Globin isolated from human blood exposed to sulfur mustard-$d_8$ (10 µM) was used as an internal standard.

### III.3.3 Inter-individual variation in adduct levels using the immunoslotblot assay for N7-HETE-Gua in DNA

The inter-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard was determined in blood samples taken from 10 donors by using the immunoslotblot assay. Blood samples were incubated with sulfur mustard in acetonitrile (0, 0.5, 1 and 5 µM; final concentration acetonitrile 1%) for 1 h at 37 °C and stored in 30 µl aliquots at -20 °C. DNA was isolated from the samples in two series and denatured. Each DNA sample was analyzed on two different days in the immunoslotblot assay, which resulted in 4 determinations for each donor at each sulfur mustard exposure concentration.

### III.3.4 Inter-individual variation in adduct levels using GC-MS analysis of N-alkylated terminal valine in hemoglobin

The inter-individual variation of *in vitro* sensitivity of human blood to sulfur mustard was determined in blood samples of 10 donors by using the GC-MS analysis of N-terminal alkylated valine in hemoglobin. For this purpose, blood samples (2 ml) taken from 10 donors were incubated with a solution of sulfur mustard in acetonitrile (500 µM; 20 µl) resulting in an exposure level of 5 µM. A blank sample was prepared by incubation of human blood with neat acetonitrile (20 µl). After incubation for 2 h at 37 °C, globin was isolated and the modified Edman degradation was performed (in duplicate), followed by derivatization with heptafluorobutyryl imidazole, according to the SOP (see Subsection III.2.8), using the VG 70-250S GC-MS system. Globin isolated from human blood exposed to sulfur mustard-$d_8$ (10 µM) was used as an internal standard.

III.3.5 Determination of dose-effect relationship and persistence of sulfur mustard adducts in blood of hairless guinea pigs exposed to sulfur mustard (i.v.)

Male hairless guinea pigs [400-500 g; species identification Crl:IAF(HA)BR] were purchased from Charles River Wiga GmbH (Sulzfeld, Germany). The animals were allowed to eat and drink ad libitum. They were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. The protocols for animal experiments were approved by the TNO Committee on Animal Care and Use.
The animals were anesthetized with racemic ketamine (80 mg/kg, i.m.). A small incision was made in the skin and some tissue was spliced in order to gain access to the jugular vein. Just before i.v. administration into the jugular vein, a solution of sulfur mustard in isopropanol (50 mg/ml) was diluted with saline in such a way that injection of 1 ml/kg body weight of this solution resulted in the required dose of sulfur mustard, *i.e.*, corresponding to 0.1 or 0.5 LD50 (estimated LD50 96 h, i.v.: 8.2 mg/kg). For each time point to be studied, a separate animal was exposed. After 10 min, 1 h, 1 day and 2, 3, 8, 14, 21, 28 and 56 days an animal was sacrified with an overdose of Nembutal®. Subsequently, blood was withdrawn by heart puncture. The blood samples were divided in equal parts (2-3 ml) and used for the immunoslotblot assay or for determination of the alkylated N-terminal valine adduct.
DNA was isolated and further processed according to the SOP described in Subsection III.1.7.
Globin was isolated and used for determination of the alkylated N-terminal valine adduct, according to the SOP described in Subsection III.2.8, using the Hewlett Packard GC-MS system equipped with a HP-5 column.

III.3.6 Skin exposure of hairless guinea pigs

Hairless guinea pigs were locally exposed to saturated sulfur mustard vapor for 1-8 min and biopsies were collected at various times after exposure, after euthanasia. The device as represented in Figure 1 was used for exposure of the skin. The estimated sulfur mustard concentration at 28 °C close to the skin was 1100 $mg.m^{-3}$.
In order to use less animals, a number of locations on the dorsal skin of the animal was exposed at various time points before biopsies were collected simultaneously.

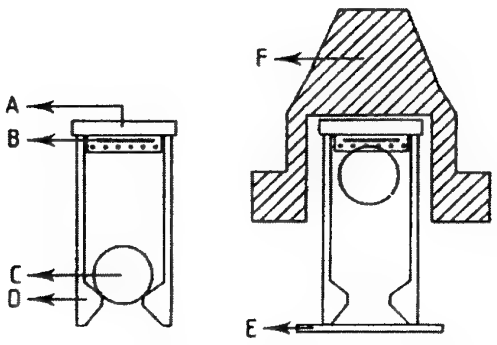


Figure 1. Schematic diagram of the device for skin exposure to air saturated with sulfur mustard vapor (Mol, 1992). At the inner side of the plastic cap (A) a piece of filter paper (B) was placed, onto which 3 µl of liquid sulfur mustard was applied. The bottom side of the glass cylinder was closed by a stainless steel ball (C). When the air in the cylinder (a volume of ca. 2 ml) had become saturated with sulfur mustard vapor (at 28 °C: ca. 1100 $mg/m^3$), the cylinder was placed onto the skin (E) and the ball was lifted with a magnet (F) for the desired period of time.

### III.3.7 Determination of persistence of sulfur mustard adducts in blood of a marmoset exposed to sulfur mustard (i.v.)

The marmoset (*Callithrix jacchus*, male, weight approximately 300 g) was anesthetized with racemic ketamine (80 mg/kg, i.m.) and sulfur mustard was administered i.v. into the jugular vein at a dose corresponding to 0.5 LD50 for the hairless guinea pig (4.1 mg/kg). Blood samples (300 µl) were drawn drawn at 1 h, 1 day and 7, 14, 21, 28 and 56 days after sulfur mustard administration, under short-lasting anesthesia with ketamine. Part of each blood sample (100 µl) was used for determination of the alkylated N-terminal valine adduct, according to the SOP described in Subsection III.2.8, using the Hewlett Packard GC-MS system equipped with an HP-5 column. The remaining part (200 µl) was used for DNA isolation.
Unfortunately, the isolated amounts of DNA were too small. Therefore, at 10 weeks after the first sulfur mustard administration the same marmoset was exposed again to the same amount of sulfur mustard and larger blood samples (550 µl) were withdrawn. In this way, sufficient DNA could be isolated. Blood samples were drawn at 1 h, 1 day and 7, 14, 21, 28, 56, 65, 70, 78, 84 and 94 days after the second sulfur mustard administration; part of each blood sample (100-200 µl) was again used for determination of the alkylated N-terminal valine adduct.

### III.3.8 *Ex vivo* exposure of human skin to sulfur mustard

The device as represented in Figure 1 was used for exposure of human skin to saturated sulfur mustard vapor. Skin was exposed for 1 min at 24 °C which corresponds to a sulfur mustard concentration of 830 $mg.m^{-3}$, *i.e.*, Ct value of 830 $mg.min.m^{-3}$.
In addition, pieces of skin (0.5 × 0.5 cm) were covered with a solution of sulfur mustard (1 ml of 0, 50 or 100 µM) in PBS containing 1% acetonitrile, for 30 min.

### III.3.9 Preparation of skin cryostat sections

After exposure, a piece of the skin was cut from the central part of the treated area and fixed in methanol/acetic acid (3/1 v/v, 1.5 h at 4 °C), rehydrated by incubation overnight in 70% ethanol at 4 °C, followed by incubation in 5% sucrose at 4 °C for 1.5 h. Next, the pieces were stretched between microscope slides and stored at –20 °C.
Alternatively, the piece of skin was immediately stretched between microscope slides, without fixation, and stored at -20 °C.
For the preparation of cryostat sections, a small piece of skin was embedded in Tissue Tek (O.C.T. compound, Miles Inc., Elkhart, USA). Subsequently, cryostat sections (5 mm thickness) were prepared at -35 °C with a cryostat microtome (2800 Frigocut, Rechert-Jung, Leica, Rijswijk, The Netherlands) on slides precoated with a solution of 3-aminopropyl triethoxysilane (2% in acetone). The slides were stored at room temperature. In the case of non-fixated skin, the cross-sections were fixated with 70% ethanol, washed with TBS (20 mM Tris HCl, 150 mM NaCl, pH 7.4) and stored at room remperature.

### III.3.10 Immunofluorescence microscopy

The procedures for the quantitative immunofluorescence microscopy of N7-HETE-Gua are essentially as described previously (Benschop and Van der Schans, 1995). Briefly, the following procedure was applied after fixation of skin cryostat sections on aminoalkylsilane-precoated slides:

- 30 min hydratation at room temperature;
- treatment with RNase (100 mg/ml) at 37 °C for 1 h;

- denaturation of the DNA with 70% formamide in 0.14 M NaCl containing 0.01 M sodium citrate at 70 °C for 5-10 min, followed by treatment with 10% formaldehyde (1 min), 70% ethanol (1 min) and washing with 50% ethanol and TBS
- treatment with proteinase K (2 mg/ml, 10 min at 37 °C);
- precoating with TBS + 5% milkpowder (30 min at room temperature);
- treatment with antibody specific for sulfur mustard-modified DNA, 2F8, in TBS containing 0.05% Tween 20 and 0.5% gelatin (overnight at 4 °C);
- treatment with a second antibody, FITC-labeled 'goat-anti-mouse', 100-fold diluted in TBS containing 0.05% Tween 20 and 0.5% gelatin, (2 h at 37 °C);
- counterstaining with propidium iodide (100 ng/ml, 10 min at room temperature).

Twin images were obtained with a laser scanning microscope (LSM-41, Zeiss, Oberkochen, Germany). The fluorescence of the FITC group and of the propidium iodide were measured consecutively to visualize the DNA in the nuclei. The fluorescein staining was used to determine the single-stranded DNA content. Images were digitized in a format of 512 × 512 pixels, the brightness of which ranges from 0 to 255 arbitrary units. The second image of the same nuclei, from the propidium iodide staining, served to localize nuclei on the image. Recognition of nuclei and calculation of the fluorescein fluorescence therein was performed with the image-processing software package SCIL-Image (CBP, Delft, The Netherlands) on a remote disk of a work station (Silicon Graphics 4D/35). On this machine, image processing, was done automatically (in batch) or in interactive way, using SCIL-image as a basic toolbox.

Alternatively, fluorescence measurements were performed using a CCD system. This consisted of a liquid-nitrogen cooled CCD camera ($LN_2$ Astromed Ltd., Cambridge, England) placed on top of a Leitz Orthoplan fluorescence microscope. The specimen was excited by light of a 100 watt dc mercury-arc lamp, filtered with a bandpass filter, adapted to the fluorochrome used (for FITC: BP 485/20). The dichroic mirror used was a Leitz DM 510. The lamp illuminated the total specimen, resulting in a fluorescence image selected by means of the emission filter BP 515-560. This image was projected onto the CCD chip of the camera using a 40× oil-immersion objective. The camera was controlled by special image pre-processing hardware (Astromed Ltd), incorporated in a personal computer (Unix). A custom-written recording program was run on this computer that allows recording of large sequences of images and is essentially similar to the one used in the set-up for the laser scanning microscopy. The images were directly transported to the same disk of the work station as in the case of the set-up for the laser scanning microscopy. The user-chosen type of image processing could be performed in the same way as for the laser scanning microscopy.

III.3.11 Applicability of the SOP for immunochemical analysis of N7-HETE-Gua in the U.S. Army Medical Research Institute of Chemical Defense

*Set up of the assay at MRICD*
Specific devices and materials were transferred from TNO-PML to MRICD (antibodies, DNA calibration samples, blocking powder, Enhanced Chemiluminescence Blotting Detection System, Puregene DNA isolation kit, blotting manifold, nitrocellulose filters). All other materials and devices were available at MRICD.

*Luminometer*
The luminometer used was a Microlite ML3000 Microtiter Plate Luminometer (Dynatech Laboratories, Inc. Chantilly, VA, USA). This luminometer is originally not intended to be used for readings on nitrocellulose filters. Since this device detects the chemiluminescence on the

upper side of the plate, we fixated the nitrocellulose filter, sealed in sheets, in the proper position on top of a white microtiter plate and placed it in the luminometer.

*Immunoslotblot assay with calf thymus DNA calibration samples*
On day 1, the thermal denaturation of calibration samples was carried out according to the SOP. In addition, these samples were further diluted (to 5 µg DNA/ml) and brought onto the nitrocellulose filter on the blotting manifold. After adsorption of the DNA to the filter, the filter was washed, air dried and crosslinked with UV-crosslinker (50 mJ/m$^2$; settings: energy: 50 mJ, time: 30 s; UV Stratalinker 1800, Stratagene, La Jolla, CA, USA).
Next, the filter was incubated with blocking solution and washed 3 times with PBS+0.1% Tween 20 after 45 min. A vortex was used since a shaking plate was not available. The filters covered with solution were vortexed at the slowest speed, making sure that all spots are washed approximately to the same extent. Subsequently, the 1$^{st}$ antibody was added and incubated overnight at 4 °C under continuous shaking (vortex). Next day, the filter washing was repeated 4 times, the last 3 times during 15 min each. Then, the 2$^{nd}$ antibody was added and the filter was incubated for 2 h at room temperature under continuous shaking (vortex), followed by 4 filter washings, the last 3 times during 15 min each.
Subsequently, free (wash) solution was removed from the filter with filter paper, the filter was placed in a closely fitting box and 10 ml of substrate solution (solution A pre-incubated at 25 °C and the mixture of solution A and B pre-incubated at 25 °C for at least 30 min) was added. Next, the filter was removed from the substrate and immediately wrapped in plastic and sealed at the three remaining sites. The wrapped filter was fixated with tape on top of the white microtiter plate and placed in the luminometer. The chemiluminescence was measured according to the required program. Data were collected in a file and the level of N7-HETE-Gua/10$^7$ nucleotides was calculated for each sample (in an Excel sheet).

*Variation in UV crosslinking dose*
Since the effectiveness of the crosslinking with the local UV crosslinking device was not known, we applied various UV doses. The UV dosing, as described in the previous paragraph, was applied 0, 1, 2 or 3 times to various parts of one nitrocellulose filter on which sulfur mustard-exposed calibration DNA was spotted. It appeared that the use of twice the indicated UV dose resulted in the highest chemiluminescence.

*DNA isolation from human skin samples (exposed to sulfur mustard at TNO-PML)*
On day 1, human skin samples exposed to sulfur mustard after removal of the fat layer (at TNO-PML) were treated with dispase overnight at 4 °C. Next day, the epidermis was transferred to an Eppendorf tube and Cell Lysis solution was added.
On day 3, the epidermis cells were completely lysed. They were further processed in the DNA isolation procedure. After DNA precipitation, hardly any pellet was observed. Nevertheless, 0.1 TE (50 ul) was added to solve the DNA overnight at room temperature. Furthermore, the DNA isolation from skin samples was repeated.

*DNA isolation from human blood samples (exposed to sulfur mustard at TNO-PML)*
On day 2, the first DNA isolation was started (from blood samples exposed to sulfur mustard at TNO-PML). Due to USA regulations, handling of blood was only allowed for US citizens registered as persons vaccinated against hepatitis B. Therefore, the RBC lysis was carried out by a US research assistant under supervision of the Dutch research assistant. Further steps were carried out by the Dutch research assistant. DNA isolation for all samples was carried out with the Puregene DNA isolation kit (Biozym) and with the Masterpure™ Complete, DNA and RNA isolation Kit (Epicentre Technologies, Madison WI, USA), which should be regarded as the US alternative of the kit used in The Netherlands. After the DNA precipitation and washing of the

precipitate, the samples were air dried and dissolved overnight at room temperature in 0.1TE buffer (50 µl) under continuous shaking.
Next day, the DNA concentration was determined using a UV/VIS spectrophotometer (Beckmann, LA, CA, USA). Then, DNA was denatured as described in the SOP and stored at -20 °C until day 4.

*DNA isolation from human blood samples (exposed to sulfur mustard at MRICD)*
On day 3, fresh human blood was exposed to sulfur mustard (0, 0.5, 1 and 5 µM for ca. 4 h at room temperature). Sulfur mustard was used from a stock diluted in acetonitrile (63 mM sulfur mustard). This was further diluted to 1 mM in acetonitrile and then added to the blood to the appropriate concentration (2 ml blood and 10 µl of 1 mM sulfur mustard resulting in 5 µM sulfur mustard, etc.). After exposure, blood was frozen at –20 °C. On day 4, DNA was isolated according to the SOP using both DNA isolation kits.

*DNA isolation from a blister sample of a possible sulfur mustard victim (received from MRICD)*
A blister sample was obtained from an individual who was possibly contaminated with sulfur mustard on his left knee. The knee had come into contact with a chlorine-containing solution used to decontaminate a sulfur mustard-contaminated floor. One day after the supposed exposure, the blister was cut off. On day 3, we started lysis of cells, after disruption of the blister sample with a grinder in Cell Lysis buffer. A sample of the same blister was treated with Dispase overnight and DNA was isolated as described above for human skin.

*DNA isolation from a blister fluid sample of a possible sulfur mustard victim (received from MRICD)*
On day 4, we started lysis of cells, possibly present in a blister fluid sample of the same possible sulfur mustard victim. DNA was isolated as described above for human skin.

III.3.12 Applicability of the SOP for GC-MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin in the U.S. Army Medical Research Institute of Chemical Defense

*Modified Edman degradation, derivatization and GC-NCI/MS analysis at MRICD*
Specific devices and materials were transferred from TNO-PML to MRICD (modified Edman reagent, globin samples of human blood exposed to sulfur mustard or sulfur mustard-$d_8$, a CPSil 5CB fused silica capillary column). All other materials and devices were available at MRICD. On day 1, the globin samples prepared at TNO-PML were processed. Modified Edman degradation, subsequent derivatization and GC-NCI/MS were largely performed as described in the SOP (see Subsection III.2.8) with a few exceptions. The organic layer obtained after modified Edman degradation was evaporated to dryness using a nitrogen evaporator (Analytical evaporator, The Meyer, N-Evap, model 111). Since the appropriate Florisil cartridges were not available, this purification step was omitted. For practical reasons, the samples were analyzed with GC-NCI/MS on day 3. GC-NCI/MS analyses were performed using the Hewlett Packard GC-MS, equipped with a CPSil 5CB fused silica capillary column.
The correct execution of the GC-NCI/MS analysis as well as of the proper work-up and reaction conditions were checked by analyzing two standard globin samples transferred from TNO-PML. These globin samples were isolated from human blood exposed to a mixture of sulfur mustard (5 or 10 µM) and sulfur mustard-$d_8$ (10 µM).
The globin samples of the second and third series were processed and analyzed in a similar manner.

*Exposure of human blood to sulfur mustard and isolation of globin samples at MRICD*
On day 2, fresh human blood was exposed to sulfur mustard (0, 1, 5, 10 and 100 µM) for ca. 4 h at room temperature. A stock solution of 10 mg sulfur mustard/ml acetonitrile was used, which had been stored in a freezer at –20 °C (Lot number: HD-U-2325-ICD-D-101297). Immediately, a 1 mM sulfur mustard solution was prepared by adding 3.2 µl of the stock solution to 196.8 µl of HPLC grade acetonitrile. Blood was exposed to 100 µM sulfur mustard by using an appropriate volume of the stock solution. An appropriate volume of the 1 mM solution in acetonitrile was added to treat blood with 1-10 µM of sulfur mustard.

On the same day, the first globin isolations from these blood samples were started. Due to USA regulations, handling of blood is only allowed to US citizens registered as persons vaccinated against hepatitis B. Therefore, the erythrocytes were washed with saline and lysed with water by a US research assistant. Isolation of globin was carried out according to the method described in Subsection III.2.4. Globin samples were analyzed for N-alkylated terminal valine as described above.

On day 3, human blood was exposed at MRICD, using a 1 mM sulfur mustard solution in isotonic saline (0.9% NaCl in water). This solution was prepared from a stock solution of 4 mM sulfur mustard in isotonic saline (5 µl of neat sulfur mustard in 10 ml saline), which had been stored in a freezer at –70 °C (Lot number: HD-U-2325-ICD-D-140/98). The frozen contents of the 10 ml vial was thawed by rubbing it between the palms of the hands. When the sulfur mustard bead started to melt, the contents of the vial were vortexed vigorously for a few seconds in order to create a dispersion of sulfur mustard. Immediately after adequate dispersion of the sulfur mustard bead, a 1 mM sulfur mustard solution was prepared by adding 500 µl of the dispersed stock sulfur mustard solution to 1.5 ml of isotonic saline. This 1 mM sulfur mustard solution was quickly vortexed (5 s) and used instantaneously for exposure of human blood samples to 0.5, 1 and 5 µM of the agent. The samples were allowed to incubate at room temperature in the fume hood for about 4 h. Subsequently, globin was isolated as described in Subsection III.2.4. Globin samples were analyzed for N-alkylated terminal valine as described above.

## III.4 Detection of hemoglobin adducts

### III.4.1 Synthesis of Nα-Fmoc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine

Nα-Boc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine methyl ester (103 mg; 0.25 mmol), which was synthesized as described previously (Benschop and Van der Schans, 1995), was dissolved in dry HCl/ethyl acetate (1 M; 62 ml). After stirring for 3 h at room temperature, FPLC analysis showed complete conversion into a single compound with a shorter retention time. The solution was concentrated under reduced pressure. Subsequently, the residue was dissolved in methanol/water (9/1, v/v; 4.5 ml) containing 0.2 M NaOH. After 1 h, FPLC analysis showed complete conversion into a compound with a shorter retention time. The reaction mixture was neutralized with acetic acid (50 µl) and concentrated. The residue was dissolved in a mixture of dioxane and aqueous 10% $Na_2CO_3$ (1/2, v/v; 6 ml). The solution was stirred under cooling in an ice-bath and subsequently a solution of Fmoc-Cl (86 mg; 0.33 mmol) in dioxane (2 ml) was added dropwise. After stirring for 16 h at room temperature, the mixture was diluted with water (10 ml) in order to dissolve solid material and extracted with pentane (5 × 15 ml) to remove unreacted and hydrolyzed Fmoc-Cl. The aqueous layer was acidified with 20% acetic acid to pH 3.5 and extracted with $CH_2Cl_2$ (3 × 20 ml). The organic layer was dried ($MgSO_4$) and concentrated, giving a white foam (95 mg; 70% over three steps, *i.e.*, removal of Boc group, saponification, introduction of Fmoc group).

$^1$H-NMR ($CDCl_3$): δ 8.0 - 7.0 (m, 10H, H-arom., H-imid.), 4.5 - 4.0 (m, 6H, $NCH_2$, CH-α, $OCH_2$-CH), 3.5 (m, 2H, $CH_2$-OtBu), 3.1-3.2 (m, 2H, $CH_2$-β), 2.9 (m, 2H, $CH_2S$), 2.6 (m, 2H, $CH_2S$), 1.2 (m, 9H, tBu).
$^{13}$C-NMR ($CDCl_3$): 174.9 (COOH), 155.7 (NC=O), 144 - 120 (C-arom., C-imid.), 73.5 [$\underline{C}(CH_3)_3$], 66.6 ($\underline{C}H_2$-OCO), 62.4 ($CH_2$O-tBu), 53.9 (N-$\underline{C}$H-$CH_2$), 48.0 (N-$CH_2$), 34 - 33 (2× $CH_2S$).
Electrospray MS: *m/z* 538 ($MH^+$), 482 ($MH^+$ - $C_4H_8$), 161 ($H_2C$=CH-S-$CH_2$-$CH_2$-O-tBu).

III.4.2 Synthesis of peptides containing a N1/N3-HETE-histidine moiety

The following peptides were synthesized:
1. A-F-S-D-G-L-A-(N1/N3-HETE)H-L-D-N-L-K, which represents the amino acid residues 70-82 of human β-globin
2. G-K-V-G-A-(N1/N3-HETE)H-A-G-E-Y-G-A-K, which represents the amino acid residues 15-26 (+ lysine) of human α-globin
3. L-(N1/N3-HETE)H-V-D-P-E-N-F-R-L-L-G-N-V-K, which represents the amino acid residues 96-109 (+ lysine) of human β-globin.

The synthesis was carried out on a 10 μmol scale with an automated solid phase peptide synthesizer. Before introduction of the modified residue, the synthesis was stopped and a solution of Nα-Fmoc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine in NMP (0.27 mg/μl; 110 μl) was added to the resin, together with a solution of PyBOP in NMP (0.54 mg/μl; 60 μl) and a solution of NMM in NMP (40 μl NMM in 175 μl NMP; 60 μl). Subsequently, the synthesis was continued as described earlier. FPLC analysis showed the presence of one main product in each case.
Electrospray MS analysis showed the presence of the expected mass:
1. *m/z* 753.3 ($MH_2^{2+}$), 502.8 ($MH_3^{3+}$)
2. *m/z* 675.2 ($MH_2^{2+}$), 450.8 ($MH_3^{3+}$)
3. *m/z* 876.4 ($MH_2^{2+}$), 584.9 ($MH_3^{3+}$), 439.1 ($MH_4^{4+}$)

Furthermore, the sequence of the peptides was firmly established by means of tandem MS analysis.

III.4.3 Immunization of mice for generation of antibodies against synthesized haptens[2]

For each hapten three mice were immunized (i.p.) with 50 μg of antigen to which spekol was added (5-10 ml/kg). Blood samples of all mice were taken after 7 days to test the serum for antibody response against hemoglobin or keratin treated with 50 μM sulfur mustard, with a direct ELISA (see Subsection III.4.6). A positive response was not observed against sulfur mustard treated proteins after 7 days. Therefore, the mice received a second immunization with the same hapten at 4 weeks after the first immunization. After the second immunization still no positive response was observed against sulfur mustard treated proteins or the antigen itself. Nevertheless, a booster with antigen (volume up to 0.2 ml) was administered 4-12 weeks later. After 3 days the animals with the strongest immune response against sulfur mustard treated protein or the antigen itself were killed with $CO_2$ anesthesia and the blood was collected by heart puncture. A cell suspension of the spleen was prepared for the production of hybrid cell strains.

[2] The procedures described in Subsections III.4.3 - III.4.6 were also used for generation of antibodies against haptens which were derived from sulfur mustard adducts with albumin and keratin.

### III.4.4 Production of hybrid cell strains

The spleen cells of the mouse were isolated for fusion with SP2/0 plasmacytoma cells. The SP2/0 plasmacytoma cells were grown in RPMI1640-medium supplemented with 10% FCS, 1 mM sodium pyruvate, 1 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 mM ß-mercaptoethanol. Spleen cells and SP2/0 cells were washed twice in RPMI-medium without serum. Next, $10^8$ spleen cells were added to $10^7$ SP2/0 cells and centrifuged (20 min at 50*g*). The supernatant was removed and the cells were exposed to fusion conditions by brief consecutive incubations of a mixture of these cells in 41% and 25% PEG 4000 as follows. The cell pellets were resuspended for 1 min in 41% PEG-solution (0.5 ml). Next, 25% PEG-solution (0.5 ml) was added and the mixture was shaken slowly for 1 min. RPMI-medium without serum (4 ml) was added twice and the cell suspension was shaken slowly for 2 min. The cell suspension was incubated for 15-30 min at room temperature and then centrifuged (20 min at 50*g*). The supernatant was removed and the pellet resuspended in RPMI-medium with 10% FCS. The cells were seeded in a 75-$cm^2$ culture flask and incubated overnight. After 24 h of incubation, the cells were centrifuged (20 min at 10*g*) and were resuspended in complete RPMI-medium (38 ml; the same medium as used for growing of SP2/0 cells) supplemented with HAT-medium, *i.e.*, 0.1 mM hypoxanthine, 16 mM thymidine, and 0.4 mM aminopterine. Hybridomas were selected in HAT-medium because they can grow in this medium whereas SP2/0 cells do not survive; spleen cells cannot be cultured (Baan *et al.*, 1982). The cells were seeded in 96-well polystyrene culture plates in HAT-medium. Hybrid cells were cultured and refreshed in this selective HAT-medium and their supernatants were screened for specific antibody production in a direct ELISA (as described in Subsection III.4.6). Cells producing specific antibodies against sulfur mustard treated proteins were recloned twice by limiting dilution as will be described in the next subsection.

### III.4.5 Cloning of hybridomas by limiting dilution

Cells of the fusion mixture producing specific antibodies against sulfur mustard treated protein (hemoglobin or keratin) were counted by light-microscopy and diluted in HAT-medium to a concentration of 50, 10 and 5 cells/ml. Per well of 96-well culture plates, 0.1 ml of one of these solutions was added resulting in 5, 1 and 0.5 cell/well. The plates were incubated for eight days without refreshing the medium. Subsequently, the amount of clones per well was counted. The supernatants of wells with only one clone were tested for specific antibody activity against sulfur mustard treated hemoglobin. Clones showing a positive result were recloned once again by limiting dilution to make sure that monoclonal antibodies would be obtained.

### III.4.6 Immunoassays (ELISA) with the polyclonal antisera and hybridoma-supernatants

The polyclonal antisera and hybridoma-supernatants were tested in a direct ELISA against hemoglobin treated with sulfur mustard (0, 50, 100, 500 µM), globin isolated from sulfur mustard-treated hemoglobin, and against the immunogen itself (if available in sufficient amounts). The ELISA was performed as follows. Polystyrene 'high binding' 96-well microtiter plates were coated with adducted and non-adducted hemoglobin, keratin or peptides dissolved in water to a final concentration of 10 µg/ml or with adducted and non-adducted globin dissolved in water to a final concentration of 2.5 µg/ml. Of these dilutions 50 µl was added per well and incubated overnight at 37 °C. The plates were washed three times with PBS containing 0.05% Tween 20. Next, the plates were incubated with PBS containing 1% FCS for 60 min at 37 °C and again washed three times with PBS containing 0.05% Tween 20. The polyclonal antisera and the hybridoma supernatants were diluted 10-1,000 times and 5-100 times, respectively, in PBS with 0.05% Tween 20 and 0.1% FCS. Of these dilutions 50 µl was added per well and incubated for 60 min at 37 °C. After washing, the second antibody, viz., goat-anti-

mouse-Ig(total)-alkaline phosphatase diluted 1:1,000 in PBS containing 0.05% Tween 20, 0.5% gelatin, and 5% FCS, was added (50 µl/well) and the plates were incubated for 60 min at 37 °C. After three washings with PBS containing 0.05% Tween 20, the plates were washed once with 0.1 M diethanolamine, pH 9.8 (100 µl). A solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$; 50 µl) was added as a substrate for alkaline phosphatase and the mixture was incubated at 37 °C for 1 h.

III.5 Detection of albumin adducts

III.5.1 Isolation of albumin from plasma

Albumin was isolated from human plasma according to a procedure described by Bechtold *et al.* (Bechtold *et al.*, 1992). Thus, shortly, whole blood was collected into an EDTA-containing vacutainer and separated into red blood cells and plasma. To the plasma an equal volume of 0.5 M $CaCl_2$ was added. The mixture was incubated at room temperature overnight and then centrifuged at 900*g* for 20 min. To the supernatant were added 4 volumes of 0.9% saline. Nine volumes of an acid/alcohol mixture (made by adding 1 ml 12 M HCl to 600 ml ethanol) were added dropwise to the supernatant. The mixture was incubated at 37 °C for 30 min and then centrifuged at 650*g* for 5 min. To the supernatant was added a volume of 0.2 M sodium acetate in 95% ethanol equal to 1/10 the total volume of the supernatant. After 15 min the mixture was centrifuged at 650*g* for 5 min, the supernatant discarded, and the albumin pellet washed with acetone. The mixture was centrifuged at 650*g* for 5 min and the supernatant discarded. The pellet was then washed in diethyl ether, centrifuged and allowed to dry overnight. Yields: 50-60 mg/ml plasma.
Analysis with SDS PAGE showed coelution with commercially available human serum albumin.

III.5.2 Tryptic digestion of albumin

Prior to tryptic digestion the disulfide bridges were reduced with dithiothreitol and the resulting thiol functions were carboxymethylated. Dithiothreitol (5 mg) was added to a solution of albumin (3 mg) in a buffer (300 µl) containing 6 M guanidine.HCl, 100 mM Tris.HCl and 1 mM EDTA, pH 8.3 (with 2 M NaOH), and the solution was incubated at 55 °C for 40 min. Subsequently, iodoacetic acid (sodium salt; 10 mg) was added and the mixture incubated at 40 °C for 30 min. The clear solution was transferred into a Slide-a-Lyzer cassette (0.1-0.5 ml) and the solution dialyzed against aqueous $NH_4HCO_3$ (3 l) for 16 h. Trypsin (2% w/w) was added and the mixture was incubated at 37 °C for 4 h. Albumin samples isolated from human blood according to the procedure described in Subsection III.5.1 gave similar HPLC chromatograms after tryptic digestion when compared to a commercially available albumin sample.

III.5.3 Synthesis of the sulfur mustard adduct of T5 of albumin

To a solution of S-HETE-cysteine (1 mmol; 225 mg) in dioxane/water (5 ml; 1/1, v/v) was added Fmoc-Cl (1 mmol; 260 mg) and $Na_2CO_3$ (270 mg) under stirring at 0°C. After 4 h, stirring was continued at room temperature for 16 h. The solution was washed with petroleum ether 60-80 and the aqueous layer was acidified (pH 3) with 1 M $KHSO_4$ (20 ml). The aqueous layer was extracted with ethyl acetate (2 × 20 ml). The organic layers were collected, dried ($MgSO_4$) and concentrated, giving a colorless oil (400 mg; 89%). FPLC analysis showed the presence of one main compound. This compound was used without further purification for the solid phase synthesis of the sulfur mustard adduct of the T5 tryptic fragment of albumin, *i.e.*, A-L-V-L-I-A-F-A-Q-Y-L-Q-Q-(S-HETE)C-P-F-E-D-H-V-K. After splitting from the resin one

main compound resulted according to FPLC analysis. This compound was used for immunochemical experiments and as reference for tandem MS experiments and HPLC analyses.
Electrospray MS: *m/z* 1269.8 ($MH_2^{2+}$), 847.0 ($MH_3^{3+}$), 635.5 ($MH_4^{4+}$).

III.5.4 LC-tandem MS analyses in tryptic digests of albumin

A PRP-1 column (length 25 cm; i.d. 0.3 mm) was used in the LC system. Eluent A consisted of water/acetonitrile, 95/5, containing 0.5% formic acid and eluent B consisted of water/acetonitrile, 2/8, containing 0.5% formic acid. The following flow scheme was applied: 100% eluent A at a flow of 0.1 ml/min from 0-5 min and, subsequently, 100% eluent A to 100% eluent B at a flow of 0.3 ml/min from 5-90 min. Flow rates were reduced by a pre-injector split: 3 to 10 µl/min from 0-5 min and, subsequently, constant at 10 µl/min. The LC column was directly connected to the electrospray probe. The injection volume was 10-40 µl. Analyses with the VG triple quadrupole mass spectrometer were performed in the multiple reaction monitoring (MRM) mode (transition $MH_3^{3+} \rightarrow$ *m/z* 1071.0, 1014.5 and 978.5). Operating conditions were: cone voltage 35 V, collision energy 12 eV, argon pressure $5 \times 10^{-3}$ mB, dwell 1.5 s/channel, span *m/z* 0.2, resolution MS1 and MS2 10. Full scan tandem-MS spectra were acquired with the Q-Tof-MS.

III.5.5 Acidic hydrolysis of albumin and derivatization with Fmoc-Cl for subsequent analysis with HPLC with radiometric detection

Albumin from blood which had been exposed to [$^{14}$C]sulfur mustard (1 mM; spec. act. 15 mCi/mmol) was hydrolyzed with 6 N HCl. To this end, the albumin sample (14 mg) was dissolved overnight in 6 N HCl (1 ml). The solution was transferred to a vacuum hydrolysis tube and the vial was carefully rinsed with 6 N HCl (1 ml). The tube was cooled in liquid nitrogen and after solidification of the contents, the tube was evacuated. Subsequently, the tube was heated at 110 °C. After 24 h, the solution was concentrated under vacuum and the residue was coevaporated with water (3 × 1 ml) to remove traces of hydrochloric acid.
A small part of the hydrolysate (1/20) was dissolved in borate buffer (0.2 M; pH 7.8; 1.5 ml). Subsequently, a solution of Fmoc-Cl in acetone (15 mM; 1.5 ml) was added and the sample was shaken vigorously for 1 min. The sample was then washed with hexane (5 × 1 ml) and the aqueous layer was used for HPLC analysis with radiometric detection.

III.5.6 Oxidation of the sulfur mustard adduct of T5 of albumin

A small amount (50-100 µg) of the synthesized sulfur mustard adduct of tryptic fragment T5 of albumin (see Subsection III.5.3) was dissolved in $H_2O$/acetonitrile (1/4, v/v; 250 µl). A mixture of acetic acid/30% $H_2O_2$ in water (1/1, v/v; 20 µl) was added and the mixture was incubated at room temperature. After 2 h, FPLC analysis showed complete conversion of starting material into a compound with a slightly shorter retention time. LC-tandem MS showed a single compound with $MW_{av}$ 2569.9 Da, which corresponds with the T5 adduct containing two sulfoxide functions. For MRM the transitions $MH_3^{3+} \rightarrow$ *m/z* 1087.2, 1031.0 and 995.5 were recorded.

III.5.7 Pronase hydrolysis of the sulfur mustard adduct of T5 of albumin

To a solution of the T5 adduct (0.5 mg) in aqueous $NH_4HCO_3$ (50 mM; 1 ml) was added a solution of pronase in aqueous $NH_4HCO_3$ (50 mM; 50 µl; 6.5 mg pronase/ml). After incubation for 2.5 h at 37 °C, the mixture was filtrated through a filter with a cut-off 10 kDa with

centrifugation at 4,000*g*, in order to remove the enzyme. The filtrate was analyzed with LC-tandem MS.
Tryptic digests of albumin were subjected to cleavage with pronase in an analogous way.

III.5.8 Pronase hydrolysis of albumin and LC-tandem MS analysis of (S-HETE)Cys-Pro-Phe

To a suspension of albumin (3 mg) in aqueous $NH_4HCO_3$ (50 mM; 750 µl) was added a solution of pronase (1 mg) in aqueous $NH_4HCO_3$ (50 mM; 100 µl). After incubation for 2.5 h at 37 °C, the mixture was filtrated through a filter with a cut-off 10 kDa with centrifugation at 4,000*g*, in order to remove the enzyme. In this case, the tripeptide (S-HETE)Cys-Pro-Phe is determined by MRM of $MH^+$ (*m/z* 470) → *m/z* 105 which corresponds with a fragment of thiodiglycol. Operation conditions were: cone voltage 30 - 35 V, collision energy 20 eV and argon pressure $3\text{-}4.10^{-3}$ mB. The injection volume was 40 µl. The LC system comprised a PRP-1 column (length 0.4 m., i.d. 0.3 mm). Gradient elution using $H_2O/CH_3CN$ 95/5 with 0.5% HCOOH and $H_2O/CH_3CN$ 2/8 with 0.5% HCOOH as eluent A and B, respectively was performed as follows,

| Time (min) | Flow (ml/min) | % Eluent A | % Eluent B |
|---|---|---|---|
| initial | 0.1 | 100 | 0 |
| 5 | 0.5 | 100 | 0 |
| 90 | 0.5 | 0 | 100 |

Flow rates were reduced by means of an LC Packings splitter (Amsterdam, The Netherlands) which was placed before the injection valve: 0-5 min, 2 → 10 µl/min and subsequently 10 µl/min.

III.5.9 Improved procedure for determination of (S-HETE)Cys-Pro-Phe with LC-tandem MS

The procedure for analysis of the adducted tripeptide (S-HETE)Cys-Pro-Phe was improved by means of:
- *Sep-pak C18 clean-up of the sample.* A Sep-pak C18 cartridge was rinsed with MeOH (5 ml) followed by 0.1% TFA/$H_2O$ (5 ml). The filtered pronase digest was applied to the cartridge. The cartridge was rinsed with 0.1% TFA/$H_2O$ (2 ml), 0.1% TFA/10% $CH_3CN$ (2 ml), 0.1% TFA/20% $CH_3CN$ (2 ml) and finally with 0.1% TFA/40% $CH_3CN$ (2 ml). The 40% $CH_3CN$ eluate was collected, concentrated and redissolved in $H_2O$ (50 µl). The sample was now ready for LC-MS analysis.
- *Modified liquid chromatography procedure.* It was observed that application of a microcolumn with Lichrosorb RP18 material (length 0.35 m, i.d. 0.32 mm), in combination with a slightly modified gradient, improved the sensitivity of the LC-tandem MS analysis.
Gradient elution using $H_2O/CH_3CN$ 95/5 with 0.2% HCOOH and $H_2O/CH_3CN$ 2/8 with 0.2% HCOOH as eluent A and B, respectively was performed as follows,

| Time (min) | Flow (ml/min) | % Eluent A | % Eluent B |
|---|---|---|---|
| initial | 0.1 | 100 | 0 |
| 5 | 0.6 | 100 | 0 |
| 25 | 0.6 | 70 | 30 |
| 45 | 0.6 | 0 | 100 |

Flow rates were reduced by means of an LC Packings splitter which was placed before the injection valve: 0-5 min, 2 → 10 µl/min and subsequently 10 µl/min.

III.5.10 Synthesis of (S-HETE)Cys-Pro-Phe

The Fmoc derivative of (S-HETE)cysteine was synthesized as described in Subsection III.5.3. The compound was used for coupling to immobilized Pro-Phe by manual addition of the Fmoc derivative and coupling reagents to the resin (10 µmol scale). After splitting the peptide from the resin with TFA, one main compound resulted, according to FPLC analysis. $^1$H-NMR and mass spectrometric data were in accordance with the proposed structure. Yield: 6 mg. This compound was used as reference for tandem MS experiments.
Electrospray MS: *m/z* 470 ($MH^+$), 105 ($HOCH_2CH_2SCH_2CH_2^+$).

III.5.11 Synthesis of N-HETE-aspartic acid

To a solution of di-O-tert-butyl-aspartic acid (141 mg, 0.5 mmol) in $H_2O/CH_3CN$ (1/1, v/v; 5 ml; pH 7.5) was added a solution of 2-(2-tert-butyloxyethylthio)ethyl chloride (0.65 mmol) in $CH_3CN$ (1 ml) in five portions within 2 h under vigorous stirring. The pH of the reaction mixture was maintained at 7.5 by the addition of aqueous NaOH (0.25 M) with a pH-stat equipment. The mixture was vigorously stirred for another 16 h, after which TLC analysis ($MeOH/CH_2Cl_2$, 5/95, v/v) showed complete conversion of starting material into two compounds, one of which coeluted with 2-(2-tert-butyloxyethylthio)ethanol. The other compound was isolated by means of silica gel chromatography (eluent: toluene/EtOAc, 100/0 --> 96/4, v/v). LC-MS analysis of this material showed the presence of the desired N-(2-(2-tert-butyloxyethylthio)ethyl)-di-O-tert-butyl-aspartic acid (*m/z* 406.2, $MH^+$).
In order to split off the protecting groups, the fully protected material was dissolved in $TFA/H_20$ (95/5, v/v; 2 ml). The mixture was concentrated and coevaporated with dioxane (2 x 1 ml) to remove traces of TFA. Next, the crude compound was applied to a column with Dowex cation exchange resin ($Na^+$ form). The column was eluted with $MeOH/H_2O$ (1/1; 10 ml) in order to remove uncharged material and subsequently with aqueous $NH_4OH$ (2 M; 10 ml). The latter eluent was lyophilized, giving the title compound as a white solid. Yield 76 mg (64%).
$^1$H-NMR δ 2.38-2.60 (2× dd, 2H, $CH_2$-β), 2.75-2.85 (m, 6H, 2× $CH_2S$, $CH_2N$), 3.49 (dd, 1H, CH), 3.81 (t, 2H, $CH_2O$).
MS *m/z* 238 ($MH^+$), 122 ($^+NH_3CH_2CH_2SCH_2CH_2OH$), 105 ($^+CH_2CH_2SCH_2CH_2OH$).

III.5.12 Derivatization of N-HETE-aspartic acid with pentafluorophenyl isothiocyanate

To a solution of N-HETE-aspartic acid (3 mg) in 0.5 M $NaHCO_3$/isopropanol (2/1, v/v) was added pentafluorophenyl isothiocyanate (25 µl). The mixture was incubated for 2 h in a shaking device at 45 °C. After acidification, the mixture was extracted with ethyl acetate (1 ml). The organic layer was dried ($MgSO_4$) and analyzed with mass spectrometry. When the sample was directly applied on the probe, a compound with the expected molecular mass could be detected (444, $M^{+\cdot}$). Unfortunately, this compound could not be detected with GC-MS, not even after derivatization with N-methyl-N-*tert*-butyldimethylsilyl-trifluoroacemide (MTBSTFA).

III.6 Detection of keratin adducts

III.6.1 Isolation of keratin from human callus

Human callus (100 mg) was soaked in Tris.HCl buffer (5 ml, 20 mM, pH 7.4) overnight. After centrifugation (30 min, 400 rpm) the residu was stirred in a buffer (5 ml; pH 7.4) containing

Tris.HCl (20 mM) and urea (8 M). After centrifugation (30 min, 400 rpm), the residue was extracted with a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM), urea (8 M), and β-mercaptoethanol (0.1 M).
The crude keratin was purified on a G 75 column (100 × 2 cm) with a buffer (pH 7.6) containing SDS (0.5%), Tris.HCl (10 mM) and DTT (10 mM); flow, 0.25 ml/min. Appropriate fractions were collected and dialysed against water. The remaining solution was lyophilized. Representative yield: 20 mg keratin/100 mg callus.

III.6.2 Exposure of human callus to [$^{14}$C]sulfur mustard

To a suspension of human callus (70-100 mg) in 0.9% NaCl (100 µl) was added a solution of an appropriate concentration of [$^{14}$C]sulfur mustard in isopropanol (100 µl). The mixture was incubated for 6 h at 37°C. Isolation of keratin was performed as described in Subsection III.6.1.

III.6.3 Exposure of human skin to saturated sulfur mustard vapor and extraction of epidermal keratins

Human skin samples (9 × 0.25 cm$^2$) were exposed to saturated sulfur mustard vapor (0, 2, 4 or 8 min) according to the method described in Subsection III.3.6. After the exposure, the epidermis was separated from the dermis by heat shock (2 min at 60 °C, followed by 5 min at 0 °C). The epidermis was cut into 3 or 4 pieces, and transferred into a 4 ml vial. Next, low salt buffer (10 mM Tris/150 mM NaCl/3 mM EDTA/0.1% N-P40; pH 7.4; 3 ml), containing a protease inhibitors cocktail (1 tablet/12 ml buffer), was added. The mixture was shaken for 1 h at 0 °C. The epidermis was sedimented, the upper layer was discarded and replaced by high salt buffer (10 mM Tris/150 mM NaCl/1.5 M KCl/3 mM EDTA/0.1% N-P40; pH 7.4; 3 ml) containing the protease inhibitors cocktail (1 tablet/12 ml buffer). The mixture was shaken for 1 h at 0 °C. Next, the epidermis was sedimented; the upper layer was discarded and replaced by washing buffer (10 mM Tris/150 mM NaCl/3 mM EDTA; pH 7.4; 3 ml) containing the protease inhibitors cocktail (1 tablet/12 ml buffer). After shaking for 30 min at 4 °C, the epidermis was sedimented. The liquid layer was discarded and the epidermis was extracted with lysis buffer (20 mM Tris/1 mM EDTA/2% SDS/1 mM DTT; pH 7.4, 2 ml), containing protease inhibitors, under gentle shaking overnight at room temperature. The mixture was centrifuged at 5,000*g* for 5 min and the supernatant containing the keratins (0.87 mg/ml, determined with the Lowry procedure (Lowry *et al.*, 1951)) was stored at –70 °C. Before treatment with 0.5 M NaOH, the keratin solutions were dialyzed against $H_2O$ in a Slyde-A-Lyzer cassette (size: 0.1-0.5 ml).

III.6.4 Synthesis of bis-O,O-pentafluorobenzoylthiodiglycol

To a solution of thiodiglycol (0.59 mg, 0.5 µl, 4.8 µmol) in a mixture of toluene and pyridin (9/1, v/v; 500 µl) was added pentafluorobenzoyl chloride (10 µl). The mixture was incubated at 45 °C for 10 min. Next, the mixture was washed with aqueous 5% $NaHCO_3$ (200 µl) and water (200 µl) and the organic layer was dried over $MgSO_4$.
GC-MS ($EI^+$): *m/z* 298 [$M^+$ - $F_5C_6COOH$], 239 [$M^+$ - $F_5C_6COOCH_2CH_2S$], 195 [$F_5C_6CO^+$].

III.6.5 Isolation and derivatization of thiodiglycol after alkaline hydrolysis of keratin followed by HPLC analysis

Purified keratin that was exposed to sulfur mustard or [$^{14}$C]sulfur mustard was incubated for 1 h at room temperature in aqueous NaOH (5 mg keratin/300 µl of 0.5 M NaOH). After neutralization with aqueous acetic acid, liberated thiodiglycol was isolated from the NaOH treated keratin sample by ultrafiltration over Centrex UF-2 filters (molecular cut-off 10 kDa).

The filtrate was evaporated to dryness and the residue was coevaporated with toluene. Next, the residue was diluted with toluene (440 µl) and pyridin (50 µl) and pentafluorobenzoyl chloride (10 µl) was added. After 5 min at room temperature the mixture was filtrated over glass wool and analyzed by HPLC with radiometric detection.

III.6.6 GC-MS analysis of thiodiglycol after alkaline hydrolysis of keratin

Human callus was exposed to sulfur mustard-$d_8$ (10 mM), followed by isolation of keratin, as described in Subsections III.6.2 and III.6.1, respectively. This keratin-$d_8$ was used in further experiments as an internal standard. A suspension of keratin-$d_8$ in water (30 µl; ca. 30 mg/ml) was added to keratin to be hydrolyzed (5 mg/300 µl of 0.5 M NaOH). Release and isolation of thiodiglycol was performed as described in Subsection III.6.5. After concentration, toluene (200 µl), pyridin (20 µl) and pentafluorobenzoyl chloride (10 µl) were added to the residue. The mixture was heated for 45 min at 45 °C, cooled to room temperature, washed with $NaHCO_3$ (5%, 200 µl) and with water (200 µl). After drying of the organic phase ($MgSO_4$), the sample was analyzed with GC-NCI/MS. In case of keratin from callus, a VG70-250S mass spectrometer operated in the NCI mode was used. The temperature of the oven of the gas chromatograph was at 120 °C. Directly after injection of the sample, the temperature was programmed to 275 °C at 8 °C/min and subsequently kept at this temperature for 5 min. In case of keratin from human skin, the Hewlett Packard GC-MS system was used, applying the conditions described in section III.2.8. Ion chromatograms were recorded after monitoring for *m/z* 510 ($M^-$, analyte) and 518 ($M^-$, internal standard).

III.6.7 Attempted mild hydrolysis of thiodiglycol esters in keratin from callus which had been exposed to sulfur mustard

First, keratin (40 µg) isolated from human callus exposed to 10 mM [$^{14}$C]sulfur mustard, was incubated in aqueous NaOH (500 µl) at various pH values (ranging from 9 to 13), in aqueous $NH_4OH$ at pH 9 (500 µl), or in aqueous NaOH (500 µl; pH 9) to which one of the following compounds was added: SDS (0.5%), urea (1 M), histidine (10 mM), and phosphate (10 mM). Furthermore, a suspension of keratin (80 µg), isolated from callus exposed to [$^{14}$C]sulfur mustard (1 mM), in water (50 µl) was incubated with a solution of porcine liver esterase (1 ml; 10 mg/ml in phosphate buffer, pH 8) or with an aqueous solution of one of the following amines (0.5 ml; 10 mM): isopropylamine, decylamine, ethanolamine, dodecylamine, octylamine and benzylamine.
After 1 h at room temperature, the solution was neutralized with acetic acid and centrifuged at 5,000*g* over an ultrafilter (MW cut-off 30 kDa). Next, 200 µl of the filtrate was analyzed for radioactivity by liquid scintillation counting.

III.6.8 Determination of amino acid composition of keratin

The amino acid composition was determined according to a standard procedure for amino acid analyses. Briefly, a sample (ca. 1 µl) of a solution of isolated keratin in 0.1% trifluoroacetic acid (10 mg/ml) was transferred to a vacuum hydrolysis tube. After evaporation of the solvent under vacuum, the protein was hydrolyzed with 6 N HCl (300 µl) at 110 °C, After 24 h, the solution was concentrated under vacuum and the residue was coevaporated with acetonitrile/triethylamine/water (2/1/2) to remove traces of hydrochloric acid. Subsequently, the amino acids were derivatized with phenyl isothiocyanate (20 µl acetonitrile/triethylamine/water/phenyl isothiocyanate, 12/2/5/1) and the obtained mixture of phenyl thiohydantoin derivatives was analyzed with HPLC. Quantitative results were derived

from comparison with results obtained from HPLC analysis of a calibration mixture of phenyl thiohydantoin amino acids.

III.6.9 Synthesis of Nα-Boc-Nω-HETE-glutamine 1-*tert*-butylester

To a solution of Boc-Glu-OtBu (0.30 g, 1 mmol) in NMP (5 ml) was added PyBOP (0.57 g; 1.1 mmol) and NMM (120 µl; 1.1 mmol). Subsequently, a solution of 2-(2-aminoethylthio)ethanol (0.13 g, 1.1 mmol) in NMP (2 ml) was added. The reaction mixture was stirred for 4.5 h at room temperature. The mixture was taken up in $CH_2Cl_2$ (20 ml) and washed with 10% aqueous $NaHCO_3$ (3 × 10 ml), 0.1 M $KHSO_4$ (pH 5.3) and water (3 × 25 ml). The combined organic layers were dried ($Na_2SO_4$) and concentrated. The crude compound was purified by means of silica gel column chromatography (eluent gradient: methanol/$CH_2Cl_2$, 0/100 to 5/95, v/v). Fractions were analyzed with TLC (eluent: methanol/$CH_2Cl_2$, 16/84, v/v). The appropriate fractions were collected and concentrated to afford a light yellow oil. Yield: 0.142 g (34.9%).
$^1$H-NMR ($CDCl_3$): δ 1.37/1.39 [2× s, 18H, 2× C(C$\underline{H}_3$)$_3$], 1.82/2.07 [m, 2H, CH-C$\underline{H}_2$(βGlu)], 2.21 [t, 2H, $J_{H,CH2}$ = 6.74 Hz, $CH_2$-C$\underline{H}_2$(γGlu)], 2.63/2.67 (2× t, 2× 2H, $J_{H,CH2}$ = 6.15 Hz, 2× S-C$\underline{H}_2$), 3.39 (t, 2H, $J_{H,CH2}$ = 5.95 Hz, NH-C$\underline{H}_2$), 3.68 (q, 2H, $J_{H,OH}$ = 5.76 Hz, C$\underline{H}_2$-OH), 4.08 [*b*s, H, NH-C$\underline{H}$(αGlu)], 5.35 (d, H, $J_{H,CH}$ = 8.13 Hz, N$\underline{H}$-CH), 6.91 [*b*s, H, C(O)N$\underline{H}$-CH2].
$^{13}$C NMR ($CDCl_3$): δ 27.9 [6× C(C$\underline{H}_3$)$_3$], 31.8 (S-C$\underline{H}_2$), 32.5 [CH-C$\underline{H}_2$(βGlu)], 34.9 (S-C$\underline{H}_2$), 39.0 (NH-C$\underline{H}_2$), 46.2 [$CH_2$-C$\underline{H}_2$(γGlu)], 53.6 [NH-C$\underline{H}$(αGlu)], 61.0 (C$\underline{H}_2$-OH), 79.9/82.1 [2× C$\underline{(CH_3)_3}$], 155.9 [C$\underline{(O)}$-NH-$CH_2$], 171.4/172.5 [2× C$(CH_3)_3$OC$\underline{(O)}$].

III.6.10 Synthesis of Nα-Fmoc-Nω-HETE-glutamine

Nα-Boc-Nω-HETE-Glu-OtBu (0.142 g, 0.35 mmol) was dissolved in a mixture of TFA/water (95/5, v/v; 2 ml). After 2 h the mixture was concentrated and coevaporated with water. The residue was dissolved in a mixture of 10% aqueous $Na_2CO_3$ (2 ml) and dioxane (5 ml). Fmoc-Cl (0.118 g; 0.46 mmol was added in small portions and the mixture was stirred for 18 h at room temperature, after which the mixture was taken up in water. The aqueous mixture was washed with light petroleum (3 × 25 ml) to remove Fmoc-OH and excess Fmoc-Cl. The aqueous layer was acidified to pH 3.5 with 1 M $KHSO_4$ and extracted with ethyl acetate (3 × 25 ml). The combined organic layers were dried ($Na_2SO_4$) and concentrated. The crude compound was purified with a Sephadex LH-20 column (eluent: $CH_2Cl_2$/methanol, 2/1, v/v). Fractions were checked with TLC (eluent: $CH_2Cl_2$/methanol/acetic acid, 45/4/1, v/v/v). The appropriate fractions were collected and concentrated to afford a white foam. Yield: 93 mg (56%).
$^1$H-NMR ($CDCl_3$): δ 1.99 [m, 2H, CH-C$\underline{H}_2$(βGlu)], 2.20 [m, 2H, $CH_2$-C$\underline{H}_2$(γGlu)], 2.64 (2× t, 2× 2H, $J_{H,CH2}$ = 5.95 Hz, 2× S-C$\underline{H}_2$), 3.40 (t, 2H, NH-C$\underline{H}_2$), 3.67 (t, 2H, C$\underline{H}_2$-OH), 4.13 [t, H, $J_{H,CH2}$ = 6.84 Hz, C$\underline{H}$-$CH_2$(Fmoc)], 4.32 [d, 2H, $J_{H,CH2}$ = 6.94 Hz, CH-C$\underline{H}_2$(Fmoc)], 6.29 [d, H, N$\underline{H}$-CH(αGlu)], 7.10 (*b*s, H, N$\underline{H}$-$CH_2$), 7.23 [t, 2H, ($J_{H,C1H}$ = $J_{H,C8H}$)≈($J_{H,C3H}$ =$J_{H,C6H}$) = 7.54 Hz, $J_{H,C4H}$ =$J_{H,C5H}$ = 1.09 Hz, C2H and C7H], 7.32 [t, 2H, ($J_{H,C2H}$ =$J_{H,C7H}$)≈($J_{H,C4H}$ = $J_{H,C5H}$) = 7.54 Hz, C3H and C6H], 7.54 (t, 2H, $J_{H,C2H}$ = $J_{H,C7H}$ = 7.44 Hz, C1H and C8H), 7.69 (d, 2H, $J_{H,C3H}$ = $J_{H,C6H}$ = 7.64 Hz, C4H and C5H).

III.6.11 Synthesis of Nα-Boc-Nω-HETE-asparagine 1-*tert*-butylester

This compound was synthesized as described for the corresponding glutamine derivative (see Subsection III.6.10), starting with Boc-Asp-OtBu. Yield: 0.19 g (50%).
$^1$H-NMR ($CDCl_3$): δ 1.44/1.47 [2× s, 18H, 2× C(C$\underline{H}_3$)$_3$], 2.69/2.74 (2× t, 2× 2H, $J_{H,CH2}$ = 6.34 Hz, 2× S-C$\underline{H}_2$), 2.84 [t, 2H, CH-C$\underline{H}_2$(βAsp)], 3.06 (*b*s, H, $CH_2$-O$\underline{H}$), 3.44 (q, 2H, $J_{H,CH2}$ = 6.05 Hz, NH-C$\underline{H}_2$), 3.75 (q, 2H, $J_{H,OH}$ = 5.85 Hz, C$\underline{H}_2$-OH), 4.38 [m, H, NH-C$\underline{H}$(αAsp)], 5.72 (d, H, $J_{H,CH}$ = 6.74 Hz, N$\underline{H}$-CH), 6.62 [*b*t, H, $J_{H,CH2}$ = 5.55 Hz, C(O)N$\underline{H}$-$CH_2$].

$^{13}$C-NMR ($CDCl_3$): δ 27.9 [6× $C(\underline{C}H_3)_3$], 32.0/35.2 (2× S-$\underline{C}H_2$), 38.4 [NH-$\underline{C}H$(αAsp)], 39.1 (NH-$\underline{C}H_2$), 51.2 [CH-$\underline{C}H_2$(βAsp)], 61.1 ($\underline{C}H_2$-OH), 79.9/82.1 [2× $\underline{C}(CH_3)_3$], 155.8 [$\underline{C}$(O)-NH-$CH_2$], 170.2/170.5 [2× $C(CH_3)_3O\underline{C}$(O)].

III.6.12 Synthesis of Nα-Fmoc-Nω-HETE-asparagine

This compound was synthesized as described for the corresponding glutamine derivative (see Subsection III.6.10), starting with Nα-Boc-Nω-HETE-asparagine 1-*tert*-butylester. Yield: 0.113 g (51%).
$^1$H-NMR ($CDCl_3$): δ 2.67/2.69 (2× t, 2× 2H, $J_{H,CH2}$ = 6.05 Hz, 2× S-$\underline{C}H_2$), 2.75/2.90 [m, 2H, CH-$C\underline{H}_2$(βAsp)], 3.40 (t, 2H, NH-$C\underline{H}_2$), 3.71 (t, 2H, $J_{H,OH}$ = 6.05 Hz, $C\underline{H}_2$-OH), 4.22 [t, H, $J_{H,CH2}$ = 6.85 Hz, $C\underline{H}$-$CH_2$(Fmoc)], 4.37 [m, 2H, CH-$C\underline{H}_2$(Fmoc)], 4.51 [t, H, NH-$C\underline{H}$(αAsp)], 7.31 [t, 2H, ($J_{H,C1H}$ = $J_{H,C8H}$)≈($J_{H,C3H}$ = $J_{H,C6H}$) = 7.53 Hz, $J_{H,C4H}$ = $J_{H,C5H}$ = 1.0 Hz, C2H and C7H), 7.40 [t, 2H, ($J_{H,C3H}$ = $J_{H,C7H}$)≈($J_{H,C4H}$ = $J_{H,C5H}$) = 7.54 Hz, C3H and C6H), 7.61 (t, 2H, $J_{H,C2H}$ = $J_{H,C7H}$ = 6.65 Hz, C1H and C8H), 7.75 (d, 2H, $J_{H,C3H}$ = $J_{H,C6H}$ = 7.54 Hz, C4H and C5H).
$^{13}$C-NMR ($CDCl_3$): δ 31.7/34.9 (2× S-$\underline{C}H_2$), 37.7 [NH-$\underline{C}H$(αAsp)], 39.1 (NH-$\underline{C}H_2$), 47.1 [$\underline{C}H$-$CH_2$(Fmoc)], 51.6 [CH-$\underline{C}H_2$(βAsp)], 61.1 ($\underline{C}H_2$-OH), 67.2 [CH-$\underline{C}H_2$(Fmoc)], 120.0/125.1/127.1/127.7 (8× $\underline{C}$H-arom.), 141.3/143.8 (4× $\underline{C}$-q) 155.8 [$\underline{C}$(O)-NH-$CH_2$], 170.9 [$CH_2O\underline{C}$(O)NH], 172.9 [$\underline{C}$(O)OH].

III.6.13 Solid phase synthesis of peptides containing an Nω-HETE-glutamine or Nω-HETE-asparagine residue

The following peptides containing modified asparagine or glutamine residues were synthesized as described earlier for the peptides containing a modified histidine residue (see Subsection III.4.2):
1. G-V-V-S-T-H-(Nω-HETE)Q-Q-V-L-R-T-K-N-K
2. G-I-Q-(Nω-HETE)Q-V-T-V-N-Q-S-L-L-T-P-L-N-K
3. G-V-M-(Nω-HETE)N-V-H-D-G-K-V-V-S-T-H-E-K

Electrospray MS analysis:
1. *m/z* 1797.2 ($MH^+$)
2. *m/z* 1955.2 ($MH^+$)
3. *m/z* 1839.0 ($MH^+$)

III.6.14 Preparation of skin cryostat section and immunofluorescence microscopy

The preparation of skin cryostat sections has been described in Subsection III.3.9. Immunofluorescence microscopy of sulfur mustard-keratin adducts in skin sections was performed analogously to the procedure described for detection of N7-HETE-Gua (see Subsection III.3.10). Briefly, the following procedure was applied after fixation of the skin section with 70% ethanol on aminoalkylsilane-precoated slides and washing with TBS:
- precoating with TBS + 5% milkpowder (30 min at room temperature);
- treatment with antibody specific for sulfur mustard-exposed keratin; supernatants of up to 32 selected monoclonal antibodies in a 1:1 dilution in TBS containing 0.05% Tween 20 and 0.5% gelatin (overnight at 4 °C);
- treatment with a second antibody, FITC-labeled 'goat-anti-mouse', 75-fold diluted in TBS containing 0.05% Tween 20 and 0.5% gelatin (2 h at 37 °C);
- counterstaining with propidium iodide (100 ng/ml, 10 min at room temperature).

Twin images were obtained with a LSM-41 laser scanning microscope The fluorescence of the FITC group above the horny layer and of the propidium iodide were measured consecutively to

visualize the presence of sulfur mustard-keratin adducts in the horny layer and the DNA in the nuclei. The fluorescein staining was used to determine the presence of sulfur mustard-keratin. Adduct levels were estimated from the brightness of the fluorescence above the horny layer. The second image, from the propidium iodide staining, served to localize nuclei on the image.

# IV RESULTS

## IV.1 Development of immunochemical assays of sulfur mustard adducts to DNA as Standard Operation Procedure

### IV.1.1 Introduction

The primary aim of this study is to develop an SOP for use in the appropriate environment, *i.e.*, an immunoslotblot assay of sulfur mustard adducts to DNA in human blood and skin. To this purpose, the various steps involved in the immunochemical assay have been simplified and mimimized as much as possible for application under field conditions, in analogy with our research on a biological radiation dosimeter (Van der Schans *et al.*, 1993, 1994b). It seemed worthwhile to develop two modes of the SOP, *i.e.*, one in which sensitivity is the most important factor and another one in which the experimental time is as short as possible. For the first mode, experiments were performed in order to simplify and to speed up the procedure while maintaining maximum sensitivity (Subsections IV.1.2-IV.1.7). On the basis of these results, an SOP has been drafted. As an alternative, some modifications have been introduced to further speed up the procedure for the assay of sulfur mustard adducts to DNA in blood accepting some decrease in sensitivity and accuracy. This resulted in a shortened SOP (Subsection IV.1.9).

### IV.1.2 Isolation of DNA from white blood cells and skin biopsies

So far, DNA was isolated from white blood cells and skin biopsies as described in the final report of a previous grant (Benschop and Van der Schans, 1995). Briefly, white blood cells from blood, isolated after lysis of the erythrocytes, were lysed with 1% SDS, followed by extraction with phenol, phenol/chloroform and chloroform/isoamyl alcohol, ethanol precipitation, RNAse treatment, treatment with proteinase K, and again the same phenol/chloroform/isoamylalcohol extraction procedure followed by ethanol precipitation. The DNA concentration was determined in a 20-fold dilution of a 4-µl aliquot of the DNA solution with an uncertainty of about 5% (standard deviation).

DNA from human skin was isolated by separating the epidermis from the dermis by dispase treatment overnight, lysis of the epidermal layer and subsequently by following the same procedure as for white blood cells.

The above-mentioned procedures for the isolation of DNA from white blood cells and skin biopsies are very laborious and time-consuming. We attempted several modifications to simplify and to speed up this procedure, using various commercially available kits. The most important advantages of these modifications are the small amount of sample required (only 300 µl of blood or 10-20 $mm^2$ of epidermis) and the decrease in labor and time needed in comparison to the originally applied phenolic extraction method. Results obtained with a number of these kits are presented in Figure 2.

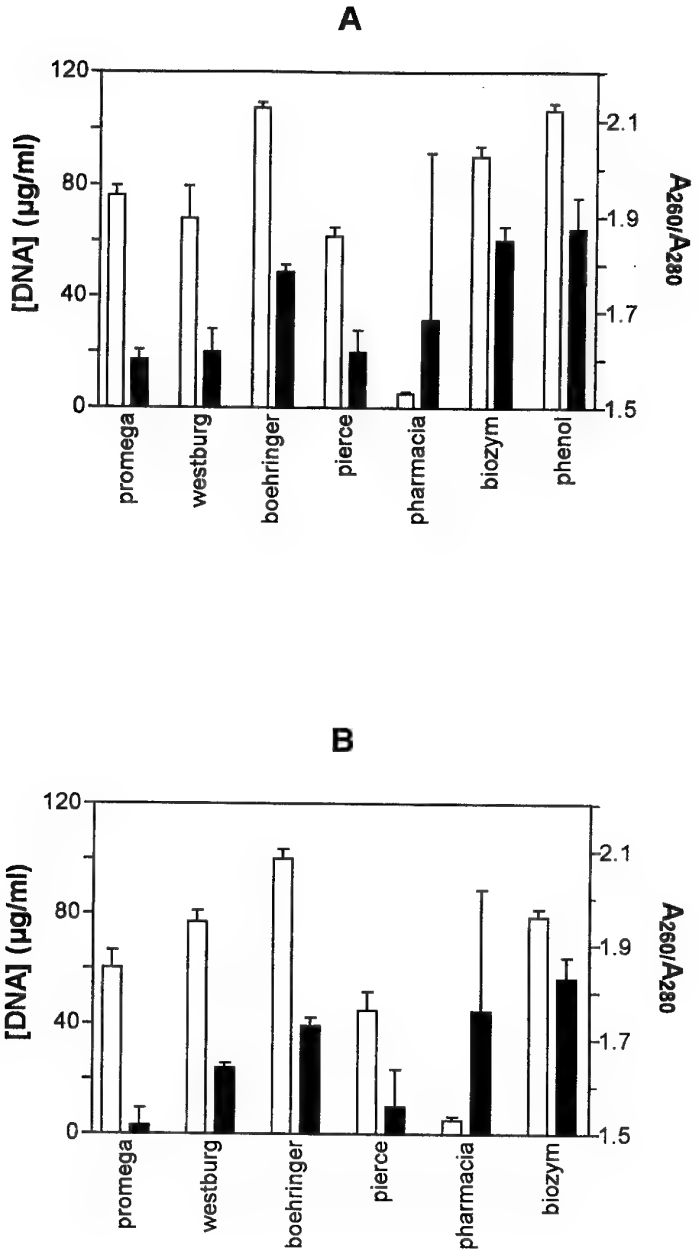


Figure 2. Yield (µg/ml final solution, open bars) and purity ($A_{260}/A_{280}$, filled bars) of DNA after isolation from human blood (300 µl) using various commercial kits (see Subsection III.1.3). DNA was dissolved in 100 µl TE buffer after the final DNA precipitation step. Panel A: fresh blood, panel B: frozen blood. All isolation procedures were carried out in quadruplicate. The data represent the mean with standard deviation. For comparison, the yield and purity of DNA are shown after isolation from fresh human blood (1 ml) using the original phenol extraction method. In the latter case the DNA was dissolved in 300 µl TE buffer after the final DNA precipitation step.

The amount of DNA obtained from the blood samples was 7-10 µg and the $A_{260}/A_{280}$ ratio[3] ranged between 1.7 and 1.9, both for fresh blood and frozen blood. Comparable results were obtained by applying the very laborious phenol extraction method to fresh blood. This method applied to frozen blood resulted in very slowly dissolving DNA pellets after the final precipitation step and extremely low yields (data not shown). The lysis of white blood cells from frozen blood was more time-consuming than that of white blood cells from fresh blood. The best results were obtained with the DNA isolation kits of Biozym and Boehringer yielding reasonable amounts of DNA and an acceptable purity.
A drawback of these procedures is the rather long period of time that is needed to lyse the cells and to dissolve the DNA precipitate. The procedure described in the SOP appeared to be optimal both with regard to lysis of the white blood cells after pelleting and with regard to dissolution of the DNA pellet.

Both the DNA isolation kits of Biozym and Boehringer were applied to blood exposed to sulfur mustard (in a range of 0.1 to 10 µM). The results indicated (data not shown) that after sulfur mustard exposure of human blood DNA can be isolated in approximately the same amounts as those obtained from unexposed blood and at a similar $A_{260}/A_{280}$ ratio (1.7 to 1.9). In general, isolation of DNA from frozen blood appeared to be more difficult than from fresh blood due to the impaired lysis of the white blood cells.

The adduct levels in the DNA samples obtained with the DNA isolation kits of Biozym and Boehringer, as detected with the immunoslotblot assay, were at least the same or even somewhat higher (20-30%) than in the DNA samples isolated in the conventional way (data not shown).

Using the commercial kit of Biozym, DNA was also isolated from human skin biopsies. To this end, the epidermis was first separated from the dermis by an overnight treatment with the enzyme dispase and then treated with the Cell Lysis Buffer following the same procedure as for white blood cells. A skin biopsy of 10-20 $mm^2$ appeared to be sufficient to yield 10-20 µg of DNA.

### IV.1.3 Variation of DNA denaturation and DNA binding conditions

DNA denaturation was carried out so far with DNA (50 µg/ml) in TE buffer containing 4.1% formamide and 0.1% formaldehyde at 52 °C for 15 min, *i.e.*, at low ionic strength. It cannot be excluded that DNA samples contain relatively more salt after the current DNA isolation procedure (as residues of previous steps) than after the originally applied phenol extraction procedure. Consequently, denaturation conditions may no longer be optimal. In order to assure an acceptable low level of salt, DNA was dissolved (after precipitation and washing) and diluted in a 10-fold diluted TE buffer (0.1TE). Variation of the concentration of formamide within a certain range (4 - 8%) did not have any effect on the extent of chemiluminescence in immunoslotblot assays on sulfur mustard treated calf thymus DNA (data not shown). At lower formamide concentration (2%) less chemiluminescence was observed. At higher formamide concentration (16%) the chemiluminescence was somewhat higher for both the DNA exposed to sulfur mustard and unexposed DNA. Therefore, the best denaturation conditions are obtained at 4% formamide and 0.1% formaldehyde content, when performed in 0.1TE. It also appeared essential to freeze the denatured DNA solution at least once before dilution with PBS and use in the immunoslotblot assay (data not shown), in order to obtain an optimal response in the immunoslotblot assay.

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[3] A higher ratio indicates a higher purity

Various UV-C doses were applied for UV-crosslinking of DNA samples in order to check the optimum dose. The results are shown in Figure 3. It is clear that the usually applied UV-C dose (50 mJ/cm$^2$) resulted in the highest response.

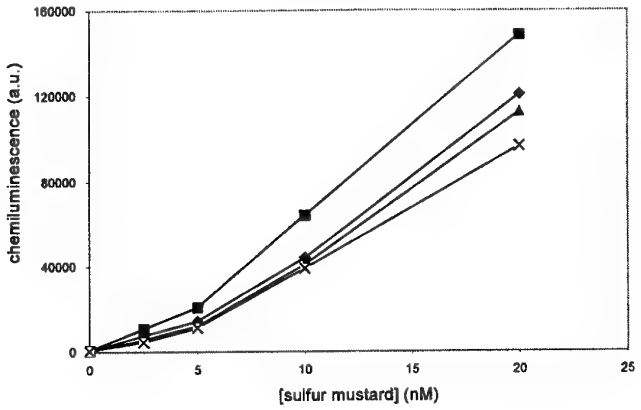


Figure 3. Immunoslotblot assay of calf thymus DNA exposed to various concentrations of sulfur mustard and blotted on nitrocellulose filters followed by cross-linking without UV exposure (▲) and with 1 × (■), 2 × (♦), and 3 × (×) a UV-C dose of 50 mJ/cm$^2$.

IV.1.4 Simplification and improvement of immunoslotblot procedure for N7-HETE-Gua

In the immunoslotblot assay, the single-stranded DNA containing N7-HETE-Gua was first slotblotted onto a nitrocellulose filter. After blotting, the slots were rinsed with PBS. Originally, the next step was baking at 80 °C in order to immobilize the DNA. In the modified protocol, the filters were dried on air and the DNA was immobilized by UV crosslinking. This modification resulted in an approximately 10-fold enhancement of the chemiluminescence signal.

The binding of DNA to the nitrocellulose filters requires a high ionic strength. This was clearly demonstrated by the absence of chemiluminescence in the immunoslotblot assay when DNA was diluted with TE buffer or water instead of PBS after denaturation. Application of a higher ionic strength than that of PBS did not result in a higher binding (data not shown).

The amount of blotted DNA appeared to be critical. Approximately, a 2-fold increase in the amount of DNA resulted in a 4-fold increase of the chemiluminescent signal (Figure 4). For that reason we decided to blot, as a standard procedure, 1 µg DNA/blot instead of various amounts of DNA as applied in previous experiments and to reserve 10 positions on the 96-blots filter for calibration samples of DNA with adduct levels in the range of 0-10 N7-HETE-Gua/10$^7$ nucleotides.

With respect to the substrate solutions of Boehringer, it appeared to be important to mix the solutions A and B and to equilibrate for 1 h at 25 °C before application on the filter (the manufacturer did not provide a clear instruction about this aspect). Since we now use a luminometer instead of cassettes with photographic film, the handling of the filters has also been modified. In this modified procedure, the filters were incubated for 1 min in substrate and

then placed in a plastic bag. Excess of liquid was pressed out and the filters were placed in the luminometer to measure chemiluminescence.

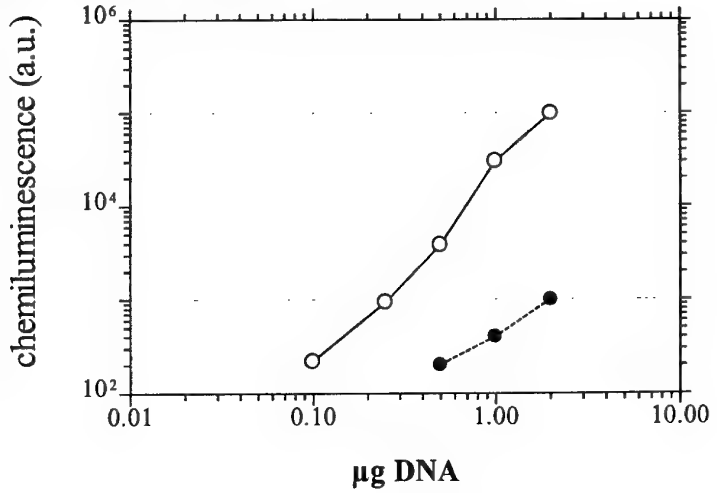


Figure 4. Immunoslotblot assay for the detection of N7-HETE-Gua in double stranded calf thymus DNA exposed to 0 (●) or 2.5 (O) nM sulfur mustard (30 min, 37 °C): dependence of chemiluminescence on the amount of DNA used in the assay. The data points represent the averages of the chemiluminescence (in arbitrary units) of two samples. Double stranded calf thymus-DNA (50 µg/ml) was made single-stranded by heating for 15 min at 52 °C in TE buffer containing 4% formamide and 0.1% formaldehyde.

The overnight incubation with the first antibody at 4 °C is the time-consuming step in the procedure. In an alternative assay, incubation with the first antibody was carried out for 2 h at 37 °C. This resulted in a lower sensitivity. For those cases in which a lower sensitivity and accuracy is acceptable, we developed a much faster SOP (see Subsection IV.1.8). In the following, we concentrated first on the development of the sensitive version.

### IV.1.5 Simplification of signal detection of the immunoslotblot procedure for N7-HETE-Gua

Originally, the chemiluminescence signal was measured by exposure of a photographic film to the blotted filters for 5-120 s. The signal was quantified by scanning of the developed film with a densitometer. Two drawbacks of this procedure were the non-linear blackening characteristics of the photographic film and the rather long time required to quantify the blackening. The purchase of a 1450 MicroBeta Trilux luminescence counter having six simultaneously operating detectors appeared to provide a significant improvement in both aspects. The response to the chemiluminescence signal over 1 s is proportional over at least 4 decades. All 96 blots are quantified within 1 min after start of the scanning. Scanning can be started immediately after placing the filters in the plastic bags and transferring these to the cassette of the device. The chemiluminescence signal is constant over a period of at least 30 min. Initial problems with the exact positioning of the filter in the cassette could be solved by adding markers on the filter. An example of a dose-effect curve is presented in Figure 5. These results clearly demonstrate the linear relationship between the chemiluminescence measured and the sulfur mustard concentration to which DNA was exposed, which could not be achieved with a photographic film. Moreover, an enhancement of chemiluminescence could be observed for double-stranded calf thymus DNA

treated with 2.5 nM sulfur mustard relative to untreated DNA, whereas the minimum detectable concentration in previous experiments was at about 10 nM sulfur mustard.

The lower detection limit in the modified assay still showed some variation which may be partly due to day-to-day variations in the state of the chemiluminescence blotting detection system. Nevertheless, it could be derived that the lower detection limit was in a range of 8-40 amol N7-HETE-Gua/blot with 1 µg DNA. This corresponds to an adduct level of 3-13 N7-HETE-Gua/$10^9$ nucleotides.

Some prudence was required in the case of assays performed with samples containing a large amount of N7-HETE-Gua. This may cause saturation of the detector system and may lead to crosstalk of chemiluminescence to the neighbouring blots (ca. 0.2%). The first problem can be solved by application of samples diluted with DNA not exposed to sulfur mustard or by application of a grey filter. The effect of crosstalk can be avoided by not using the neighbouring blots or also by diluting samples with unexposed DNA. According to the manufacturer the use of a red filter should also decrease crosstalk.

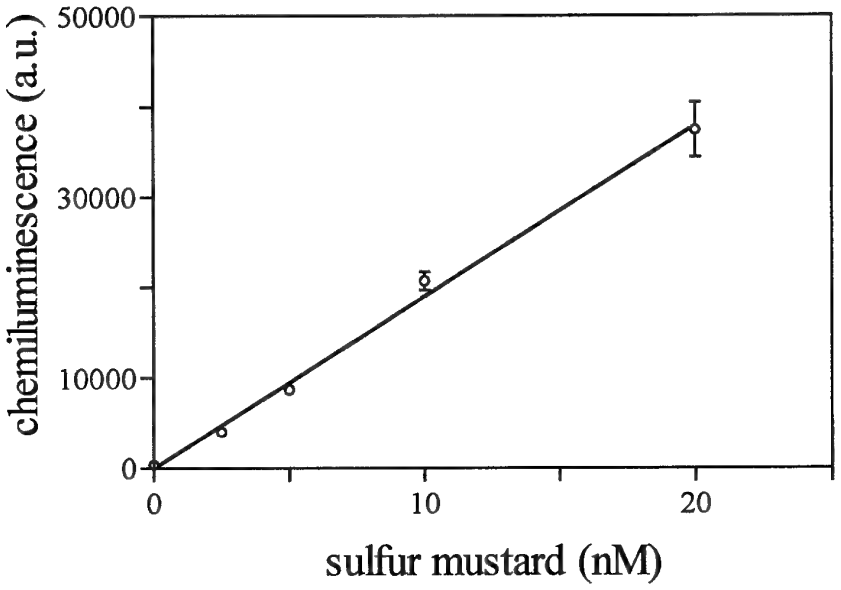


Figure 5. Immunoslotblot assay of N7-HETE-Gua in double stranded calf thymus DNA exposed to various concentrations of sulfur mustard for 30 min at 37 °C. The data points represent the average of the chemiluminescence (in arbitrary units) of two samples. The error bars represent the range between these two samples. Double stranded calf thymus DNA was made single-stranded by heating for 15 min at 52 °C in 10 mM Tris buffer containing 1 mM EDTA, 4% formamide and 0.1% formaldehyde.

IV.1.6 Effect of conditions for sulfur mustard treatment of DNA and blood on the induction of N7-HETE-Gua

At the start of this study the minimum detectable concentration of the immunoslotblot assay for exposure of human blood was 70 nM sulfur mustard. This corresponds to an adduct level of 300

N7-HETE-Gua/$10^9$ nucleotides. Since the lower detection limit for double stranded calf thymus DNA treated by sulfur mustard was substantially improved by employing the modified immunoslotblot procedure (Subsection IV.1.5), we intended to determine concentration-effect curves for *in vitro* exposure of human blood to sulfur mustard including a lower concentration range, using the same procedure. In these experiments blood was mixed with a diluted solution of sulfur mustard at room temperature and the reaction was terminated at 1 h after administration.

The data obtained (Figure 6) indicate that the levels of N7-HETE-Gua determined in blood exposed to sulfur mustard were lower than those observed previously (Benschop and Van der Schans, 1995) over the whole concentration range. Even at 100 nM sulfur mustard, N7-HETE-Gua levels were only slightly increased in comparison to those in untreated blood, in spite of the increased sensitivity of the assay. In addition, the ratio between adduct levels found in blood treated with 10 µM and 1 µM sulfur mustard appeared to be more than the expected factor of 10 (Figure 6). Therefore, some modifications were carried out to the treatment conditions of blood with sulfur mustard: (i) blood was added to the sulfur mustard dilution and *vice versa*, (ii) after mixing at room temperature the incubation mixture was left at room temperature for 1 h and placed in an incubator (37 °C), and (iii) blood collected in heparin and in EDTA was used. The results as summarized in Figure 6 indicate that addition of blood to sulfur mustard instead of sulfur mustard to blood did not eliminate the unexpected concentration-effect observed after treatment with sulfur mustard at low concentrations. Incubation at 37 °C after mixing seemed to be an improvement. The use of heparin instead of EDTA seemed to have also a positive effect on the linearity of the concentration-effect relation. However, drawbacks of the use of heparin instead of EDTA appeared to be difficulties with solving the DNA pellet after isolation of the DNA and the decreased purity as reflected in a lower $A_{260}/A_{280}$ ratio. When white blood cells (purified by lysis of the erythrocytes) were treated with sulfur mustard, the linearity was also better than with blood. These data suggest that the reaction temperature influences the linearity of the concentration-effect curve of sulfur mustard exposure of human blood.

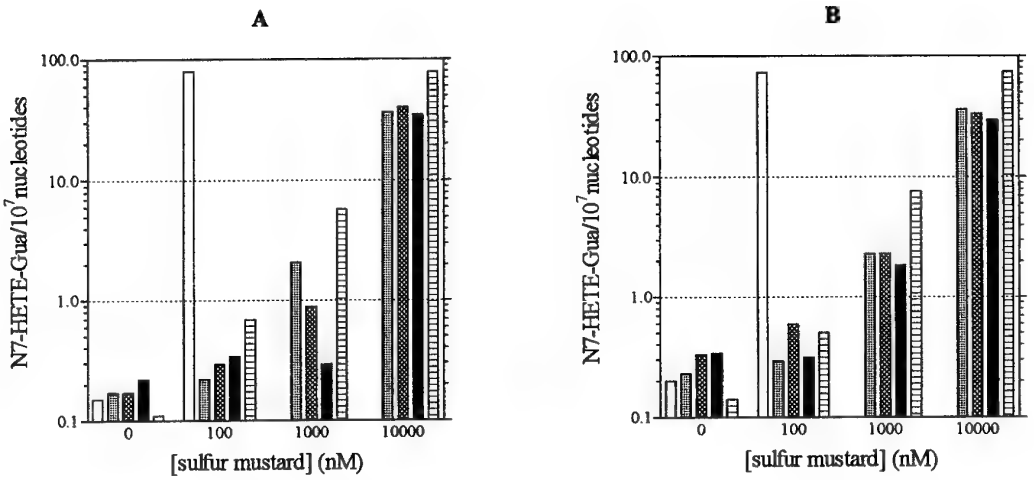


Figure 6. Immunoslotblot assay of N7-HETE-Gua in DNA of white blood cells of human blood collected in EDTA (panel A) and in heparin (panel B) that was exposed to sulfur mustard at various conditions. Human blood was mixed with an appropriate sulfur mustard dilution at room temperature and the incubation mixture was either immediately placed in an incubator at 37 °C ( ▒ ) or left at room temperature ( ■ : blood added to sulfur mustard solution; ■ : sulfur mustard solution added to blood). For comparison, double-stranded calf thymus DNA (open bars) and white blood cells (bars with horizontal lines) have been exposed to sulfur mustard at room temperature. The data represent the average of the adduct level derived from the chemiluminescence of two samples in relation to that of calibration DNA samples. The estimated error ranged from about 0.1 N7-HETE-Gua/$10^7$ nucleotides for the samples not exposed to sulfur mustard to about 5 N7-HETE-Gua/$10^7$ nucleotides for the highest concentrations.

### IV.1.7 Day-to-day variability of the immunoslotblot assay for N7-HETE-Gua in DNA in a single blood sample

As described already in Subsection IV.1.5, the lower detection limit for the detection of N7-HETE-Gua in DNA varied within a certain range due to day-to-day variations in the blotting detection system. In addition, the day-to-day variability in the level of N7-HETE-Gua in DNA measured in the same DNA sample isolated from sulfur mustard treated blood was sometimes more than 20%. Some improvement seemed possible in the DNA isolation and denaturation procedure by making the DNA solution more homogeneous through repeated freezing-thawing cycles both after solving the DNA precipitate and after the denaturation procedure. On the basis of these results an SOP has been drafted (see Subsection III.1.7).

Blood samples have been analyzed to assess a concentration-effect curve. A linear relationship was not observed between the number of N7-HETE-Gua/$10^7$ nucleotides and the sulfur mustard concentration ranging from 0.1 and 10 μM sulfur mustard. This relationship can be described with the equation

$$y = 0.4 \times [SM]^{1.4}$$

in which y and [SM] are the number of N7-HETE-Gua/$10^7$ nucleotides and the sulfur mustard concentration in µM, respectively. When using the PureGene kit of Biozym instead of the DNA isolation kit of Boehringer for DNA isolation, the equation is:

$y = 1.3 \times [SM]^{1.2}$

These results indicate that a less steep exponential relationship between the number of N7-HETE-Gua/$10^7$ nucleotides and the sulfur mustard concentration and a higher sensitivity at the lower sulfur mustard concentrations were obtained when using the PureGene kit. For that reason, the validation experiments (including those regarding the day-to-day variability) have been carried out with the PureGene kit.

The day-to-day variability has been determined by exposing human blood to 0, 0.5, 1 or 5 µM sulfur mustard. Blood samples were subsequently frozen in 30 µl aliquots. On 6 different days samples were thawed, DNA was isolated, denatured and analyzed in duplicate for the presence of N7-HETE-Gua. The variation in chemiluminescence between the duplicates through the entire chemiluminescence range (from ca 60 to 60,000 units) was only about 2%. However, the day-to-day variability appeared to be rather high as can be derived from Figure 7.

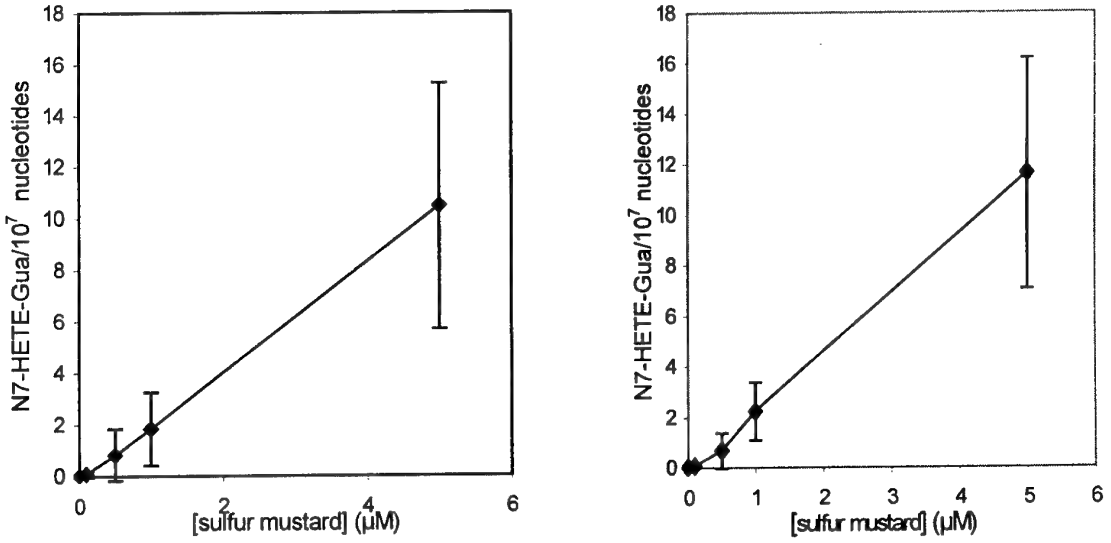


Figure 7. Day-to-day variability in N7-HETE-Gua detection in sulfur mustard-exposed human blood, frozen in 30 µl aliquots and analyzed with the immunoslotblot assay on 6 different days. The entire experiment was carried out twice (left and right panel). The data presented are the averages of 6 duplicate analyses with standard deviations.

Even when the entire procedure starting with the protein precipitation step was applied to calf thymus DNA exposed to various sulfur mustard concentrations, still a large spread in chemiluminescence was observed for the results obtained in three series performed simultaneously (a 3-fold difference between the highest and the lowest value of corresponding samples). Repeated measurements of the denatured samples in the immunoslotblot assay showed the same variation indicating that the reproducibility of the immunoslotblot assay itself

is satisfactory. Consequently, the largest variation is induced during DNA isolation and/or denaturation.

As can be derived from Figure 5 (Subsection IV.1.5), a 10% deviation in DNA concentration resulted in about 20% deviation in chemiluminescence. Repeated measurement of the DNA concentration showed a variation of 10% at an $A_{260}$ value of 0.25.

Another source of uncertainty might be the procedure of sampling on the filter and the procedure of incubation of the filter in 1st and 2nd antibody solution and in the washing solution. In order to study this aspect, an aliquot of the same sulfur mustard-exposed solution of calf thymus DNA was spotted on all 96 places on the filter and further processed in the immunoslotblot assay. It appeared that the chemiluminescence was not equally distributed over the various spots: the lowest values were observed in the middle of the filter (a 1.7-fold difference was observed between the highest and lowest values). Starting the vacuum pump after adding the last sample or applying a less strong underpressure did not result in an improvement. Application of 3 instead of 2 layers of filter paper carrying the filter appeared to be an improvement but was still not sufficient. Slowly shaking of 1st and 2nd antibody solution in a larger volume produced a more constant flow of liquid over the filter, resulting in an equal distribution over all the spots. This turned out to be the most important improvement.

### IV.1.8 Standard Operating Procedure for the immunoslotblot assay of sulfur mustard adducts to DNA in blood and skin samples

On the basis of the results obtained, an SOP for an immunoslotblot assay of sulfur mustard adducts to DNA in blood and skin samples has been drafted in which the problems mentioned in the previous subsection have been avoided. The SOP is described in detail in Subsection III.1.7.

### IV.1.9 Development of a shortened Standard Operating Procedure for the immunoslotblot assay

The SOP described in Subsection III.1.7 is rather time-consuming, mainly due to the time needed to dissolve the DNA pellet (overnight) and the overnight incubation of the nitrocellulose filter with the 1st antibody. In order to speed up the procedure while accepting some loss of sensitivity and accuracy, some steps in the procedure were omitted, shortened or carried out in a different way. This resulted in a shortened SOP, described in detail in Subsection III.1.8. According to this procedure, the assay could be carried out within 9 h, whereas up to 12 blood samples could be analyzed.

We applied this shortened procedure to blood samples of two donors exposed *in vitro* to sulfur mustard. The chemiluminescence of both the calf thymus DNA samples used for calibration as well as the DNA samples isolated from the blood samples was ca. 10-fold smaller than that of the corresponding samples assayed in the usual way (see Subsections IV.3.2 and IV.3.4). Moreover, the DNA of the unexposed blood sample of one donor (#9) showed some aspecific chemiluminescence. Nevertheless, a similar dose-effect relationship was observed as with the comprehensive SOP (Figure 8). From these data, it can be derived that *in vitro* exposure of human blood to 1 μM sulfur mustard is still detectable with the shortened SOP.

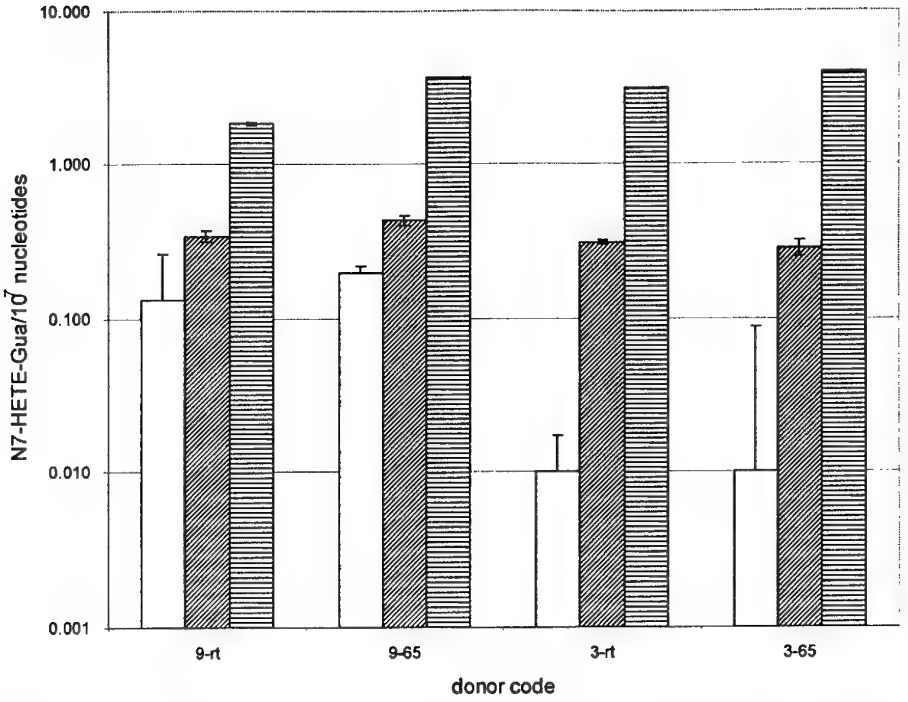


Figure 8. Immunoslotblot assay according to the shortened SOP of N7-HETE-Gua in DNA of human blood of 2 donors (coded 3 and 9) exposed *in vitro* to 0 (open bars), 1 μM (slashed bars) and 5 μM (bars with horizontal lines) of sulfur mustard for 1 h at 37 °C; rt: DNA dissolved at room temperature, 65: DNA dissolved at 65 °C. In the unexposed blood samples of donor 3, sulfur mustard adducts were not observed.

## IV.2 Development of a GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin as a Standard Operating Procedure

### IV.2.1 Introduction

A number of experiments were carried out to optimize the procedure for a modified Edman degradation with respect to sensitivity and simplicity, as part of the development of an SOP for the determination of sulfur mustard adducts to the N-terminal valine in hemoglobin. On the basis of these results, an SOP has been drafted. $^{14}$C-labeled sulfur mustard was advantageously used in some of these experiments as well as in experiments described in other sections of this chapter. Results of the synthesis of the compound are given in this section. In addition, the results are presented of the analyses of blood samples of nine Iranian victims from the Iran-Iraq conflict, who were treated in the Academic Hospital of Utrecht, by using the original modified Edman degradation procedure (Benschop and Van der Schans, 1995).

### IV.2.2 Synthesis of [$^{14}$C]sulfur mustard

In the final report of the previous grant (Benschop and Van der Schans, 1995) we reported that the synthesis of [$^{35}$S]sulfur mustard was troublesome. The yield and the purity of the obtained product varied considerably for various synthetic runs. The bottle-neck of the synthesis was

probably the purity of the [$^{35}$S]hydrogen sulfide. Therefore, we focussed our attention on the synthesis of [$^{14}$C]sulfur mustard, which has the additional advantage of a long half life of the $^{14}$C-isotope.

As starting material we chose commercially available [$^{14}$C]bromoacetic acid. Reduction with borane tetrahydrofuran complex solution in THF afforded 2-bromo-[1-$^{14}$C]ethanol, which was used without further purification (Figure 9). Reaction of the latter with $Na_2S$ afforded [$^{14}$C]thiodiglycol in moderate yield, which could be isolated by silica gel column chromatography. The major disadvantage of this procedure was the concomitant formation of the $^{14}$C-labeled disulfide of mercaptoethanol, which could not be removed easily by silica gel column chromatography since this compound has a similar retention as thiodiglycol. This problem could be circumvented by reaction of [$^{14}$C]bromoethanol with 2-mercaptoethanol under the agency of sodium ethylate; the disulfide which was formed in this case was not radioactive. [$^{14}$C]Thiodiglycol was obtained in 54% yield. Finally, conversion of [$^{14}$C]thiodiglycol into [$^{14}$C]sulfur mustard was effected by reaction with thionyl chloride. The crude sulfur mustard was contaminated with a radioactive compound with a longer retention time upon GC analysis. In order to isolate [$^{14}$C]sulfur mustard, the crude sample (obtained from 0.27 mmol [$^{14}$C]thiodiglycol) was diluted with cold sulfur mustard (0.2 mmol) and then distilled. Two batches of [$^{14}$C]sulfur mustard were obtained with a radiochemical purtity > 99% and a specific activity of 15 mCi/mmol.

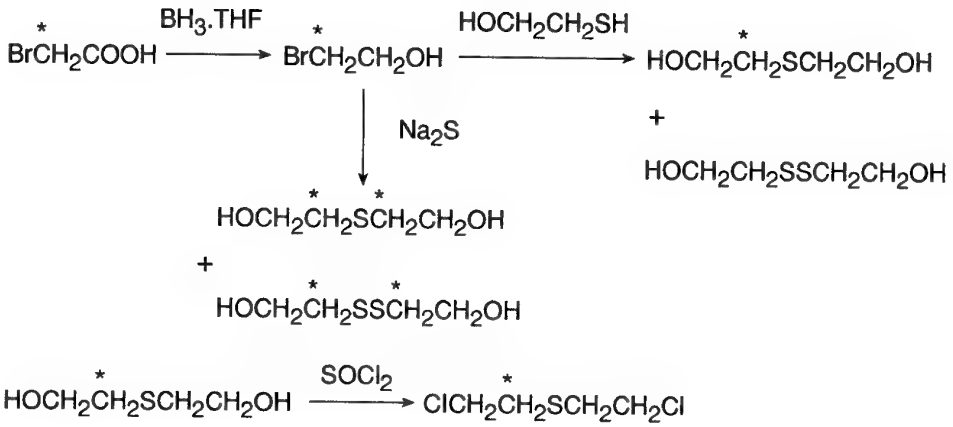


Figure 9. Synthesis of [$^{14}$C]thiodiglycol containing either one or two radioactive labels (C*) and the subsequent formation of [$^{14}$C]sulfur mustard containing one radioactive label.

Although this procedure is more reliable than the synthetic procedure previously used for synthesis of [$^{35}$S]sulfur mustard, the conversion of [$^{14}$C]thiodiglycol into [$^{14}$C]sulfur mustard by reaction with thionylchloride is still somewhat troublesome. The crude [$^{14}$C]sulfur mustard thus obtained had to be diluted with cold sulfur mustard and had to be distilled, leading to a product with relatively low specific activity (15 mCi/mmol) in low yield. The synthesis of [$^{14}$C]sulfur mustard was improved (Fidder *et al.*, 1999) by isolation of the intermediate 2-bromo-[1-$^{14}$C]ethanol before conversion into [$^{14}$C]thiodiglycol and by treatment of [$^{14}$C]thiodiglycol with 12 N HCl, as reported by Ott *et al.* (1986) and by Bent (1947), instead of with thionylchloride. In a representative run, [$^{14}$C]thiodiglycol was obtained in 79% yield. The formation of [$^{14}$C]sulfur mustard proceeded in good yield (70%; 56% overall, starting from bromo[1-

$^{14}$C]acetic acid), having a specific activity of 56.4 mCi/mmol and a chemical and radiochemical purity of 99%, as assessed by GC analysis. Further purification was not required.

### IV.2.3 Simplification of the modified Edman procedure

The first step in the analysis of adducted N-terminal valine in hemoglobin is the isolation of globin. In an attempt to shorten the procedure by leaving out this isolation step, hemolysates of human blood that was exposed to 10 µM of sulfur mustard were treated with the modified Edman reagent. However, sulfur mustard adducts could not be detected by GC-NCI/MS analysis of the samples obtained after further processing of the treated hemolysates in the usual way.

Next some modifications were introduced into the modified Edman procedure itself in order to simplify and shorten the procedure. The degradation step was performed by reaction for 2 h at 60 °C instead of incubation overnight at room temperature followed by reaction for 2 h at 45 ° C. Furthermore, the reaction mixture was worked up by extraction with toluene only, leaving out the first extraction step with diethyl ether. Both the original and the simplified procedure were used for processing of globin which had been isolated from blood exposed to $^{14}$C-labelled sulfur mustard (1 mM). Identical results were obtained upon HPLC analysis with radiometric detection.

### IV.2.4 Enhancement of the sensitivity of the modified Edman procedure

Two approaches were followed in order to lower the minimum detectable concentration for the modified Edman procedure. In the first approach, GC-NCI/MS of the final sample was performed using a TCT injection technique. As a preliminary step for such a TCT injection, the sample was applied onto Tenax absorption material. After venting most of the solvent by a stream of helium, the analytes are thermally desorbed and transferred into a cold trap. The analytes are injected onto the analytical column by flash heating of the cold trap. Much larger sample volumes (*e.g.*, 50-100 µl) can be used with this injection technique than with a normal injection (sample volume 1-3 µl). The detection limit of synthesized thiohydantoin of N-HETE-valine derivatized with a heptafluorobutyryl group was determined to be 100 fg when a sample volume of 50 µl was applied. By using this injection technique (sample volume 50 µl), the minimum detectable concentration of the modified Edman procedure for exposure of human blood to sulfur mustard was lowered from 100 to 30 nM of the agent. However, analysis in which the TCT injection technique was applied could not routinely be performed, since the results were not sufficiently reproducible.

In the second approach, attempts were made to lower the minimum detectable concentration by purification of the crude thiohydantoin obtained after the modified Edman degradation, by means of solid phase extraction procedures. Firstly, Florisil cartridges containing straight phase silica gel were used, since it is advantageous to obtain the samples as solutions in an anhydrous, apolar solvent for introduction of the heptafluorobutyryl group. Globin samples were used which had been isolated from blood exposed to $^{14}$C-labeled sulfur mustard (1 mM). The purification step was followed by HPLC with radiometric and UV detection. Only a minor loss (< 2%) of the thiohydantoin was observed. The UV pattern demonstrated that a significant purification could be obtained by inserting this relatively simple purification step (see Figure 10). A purification with Sep-Pak C18 gave a similar outcome, although the loss of thiohydantoin was higher (13%). Combining the two purification steps afforded an even more purified sample.

Since it may be expected that a lower minimum detectable concentration may be achieved by applying these purification steps, attempts were made to detect exposure to 10 nM of sulfur mustard, using the simplified version of the modified Edman procedure (Subsection IV.2.3). However, the Edman derivative could not be detected either after applying purification with Sep-pak C18 cartridges or Florisil cartridges nor after applying both purification steps.

Application of these purification steps may also allow to process larger batches of globin without accumulation of impurities in the final sample. Consequently, lower exposure levels can be detected. Best results were obtained when globin samples of up to 60 mg were used, applying the simplified version of the modified Edman procedure. When large amounts (up to 500 mg) of globin were used, impurities present in the sample prevented proper analysis. In addition, the sample to be analyzed by GC-MS was concentrated from 100 µl to ca. 30 µl which led to a more pronounced peak in the chromatogram upon GC-MS analysis. Further concentration led to disturbance of the peakshape, probably by accumulation of impurities. However, these modifications did not result in a significant lower minimum detectable concentration, *i.e.*, in the proper analysis of the Edman derivative in samples obtained from blood that had been exposed to sulfur mustard at concentrations lower than 100 nM. It can also be concluded from these results that ca. 60 mg of globin should be processed in case of low exposure levels provided that sufficient globin is available.

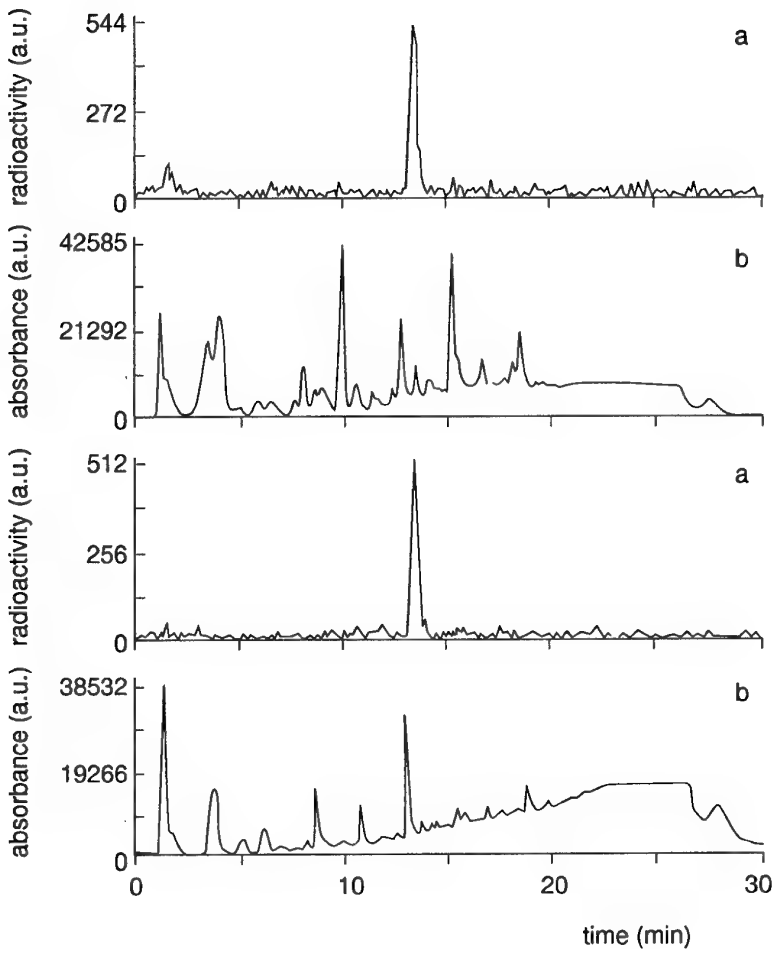


Figure 10. HPLC chromatogram (PepRPC 5/5 column) of thiohydantoin obtained after modified Edman degradation of globin isolated from human blood that was exposed to [$^{14}$C]sulfur mustard (1 mM), before (2 upper panels) and after solid phase extraction with a Florisil cartridge (two lower panels). A, detection of radioactivity; B, UV detection (254 nm). Eluent: 0.1% TFA in water with a linear gradient to 0.1% TFA in acetonitrile/water (80/20, v/v) in 20 min.

### IV.2.5 Standard Operating Procedure for analysis of alkylated N-terminal valine residue

On the basis of the obtained results, an SOP was drafted for analysis of the alkylated N-terminal valine residue in hemoglobin by means of the modified Edman procedure, which is described in detail in Subsection III.2.8.

### IV.2.6 Day-to-day variability of the adduct level determined with the modified Edman procedure

The day-to-day variability of the modified Edman procedure was investigated in order to assess the feasibility of this assay. For this purpose, blood from one single person was exposed to sulfur mustard (5 μM). The blood sample was divided into ten portions, red blood cells were isolated and stored at -20 °C. On ten points of time, globin was isolated and subjected to the SOP for the modified Edman degradation (see Subsection III.2.8). Globin from blood which had been exposed to sulfur mustard-$d_8$ (10 μM) was used as an internal standard. The results of duplicate experiments are shown in Table 1.

Table 1. Ratios for the peak areas of analyte and internal standard determined at various time points in a single human blood sample that has been exposed to 5 μM sulfur mustard, by using the SOP for the modified Edman degradation. Globin (20 mg) from blood which had been exposed to sulfur mustard-$d_8$ (10 μM) was used as an internal standard.

| Sample | Period of storage at –20 °C after exposure (day) | Peak ratio analyte/internal standard[a] |
|---|---|---|
| 1 | 0 | 0.3 |
| 2 | 7 | 0.3, 0.4 |
| 3 | 8 | 0.4, 0.4 |
| 4 | 21 | 0.3, 0.3 |
| 5 | 22 | 0.3, 0.9 |
| 6 | 42 | 0.7, 0.8 |
| 7 | 43 | 0.3, 0.4 |
| 8 | 56 | 0.5, 1.1 |
| 9 | 63 | 0.7, 0.8 |
| 10 | 84 | 0.4, 0.4 |
| Mean ± S.D. | | 0.5 ± 0.2 |

[a] Duplicate experimens, except for sample #1.

A rather larger difference between the duplicate results was observed for two samples (#5 and #8). In two of the ten samples (#6 and #9), the ratios for the peak areas of analyte and internal standard were found considerably higher than in the other samples.

### IV.2.7 Analysis of nine blood samples from Iranian victims by using the modified Edman procedure

Blood samples taken in 1986 from Iranian victims from the Iran-Iraq conflict were analyzed for the presence of the sulfur mustard adduct of hemoglobin. These victims were exposed 8 – 9 days before blood sampling. Some of them had donned gas masks during part of the exposure period or the whole exposure period. The victims were transported to the Academic Hospital in

Utrecht, The Netherlands, for treatment. All patients suffered from skin injuries compatible with sulfur mustard intoxication; some of them had respiratory difficulties.

Unfortunately, red blood cells and plasma had not been separated immediately after taking the blood samples and the red cells could no longer be isolated due to lysis during storage. Therefore, a crude globin sample was obtained. The original modified Edman procedure was applied, since at the time of analysis we were not sure whether the new procedure would give reliable results. Globin from blood which had been exposed to 1 mM sulfur mustard-$d_8$ was used as an internal standard. The analyses were carried out twice, at various points of time. For the first series 20-30 mg of globin was used, for the second series 40-50 mg of globin was used. In the same series globin (20-30 mg or 30-40 mg) originating from human blood that had been exposed to 0.1, 1 and 10 µM was processed, which served as reference samples. In all samples, a significant signal originating from the thiohydantoin of N-HETE-valine could be detected (see Figure 11 for an example). The results are given in the Table 2. All ratios of the peak areas of analyte and internal standard are expressed for the use of 20 mg of globin and 10 µl of internal standard.

Table 2. Ratios for the peak areas of analyte and internal standard determined in blood samples taken from Iranian victims 8-9 days after exposure to sulfur mustard, by using the original modified Edman degradation procedure. Globin (20 mg) from blood which had been exposed to sulfur mustard-$d_8$ (1 mM) was used as an internal standard. Globin isolated from human blood which had been exposed to 0.1, 1 and 10 µM was also processed as reference samples.

| Iranian victim | Peak ratio analyte/internal standard[a] | Peak ratio analyte/internal standard[a] |
|---|---|---|
| 1 | 0.04 | 0.17 |
| 2 | 0.03 | 0.10 |
| 3 | 0.04 | 0.12 |
| 4 | 0.06 | 0.20 |
| 5 | 0.04 | 0.21 |
| 6 | 0.09 | 0.27 |
| 7 | 0.03 | 0.17 |
| 8 | 0.06 | 0.38 |
| 9 | 0.07 | 0.49 |
| Reference samples[b] | | |
| 0.1 µM | 0.01 | 0.01 |
| 1.0 µM | 0.09 | 0.03 |
| 10 µM | 1.33 | 1.14 |

[a] Modified Edman procedure carried out with 20-30 mg (second column) or 40-50 mg (third column) of globin and 5 µl (second column) or 8-10 µl (third column) of a solution of 10 mg internal standard/ml. All ratios are corrected for use of 20 mg of globin and 10 µl of the internal standard solution.

[b] Modified Edman procedure carried out with 20-30 mg (second column) or 30-40 mg (third column) of globin isolated from human blood exposed to 0.1, 1 or 10 µM of sulfur mustard; 5 µl of a solution of 10 mg internal standard/ml was used. All ratios are expressed for the use of 20 mg of globin and 10 µl of the internal standard solution.

Unfortunately, the second series of experiments performed on the blood samples of the Iranian victims at a later point in time resulted in much higher peak ratios. In this second series, twice the amount of globin was used in the modified Edman procedure, for which the results presented in Table 2 have been corrected. The reason for this discrepancy is yet unclear. Nevertheless, we can derive from these results that the exposure level of the Iranian blood samples must have been approximately 0.3-2 μM, when the values of 0.38 and 0.49 are regarded as outliers. These results are in agreement with estimated exposure levels derived from the adduct levels determined for Cys-34 in albumin isolated from the Iranian blood samples (see Subsection IV.5.4). Unfortunately, exposure to sulfur mustard could not be confirmed from analysis of N7-HETE-Gua by using an immunochemical assay because of coagulation or partial precipitation of the blood samples which hampered proper isolation of DNA from lymphocytes.

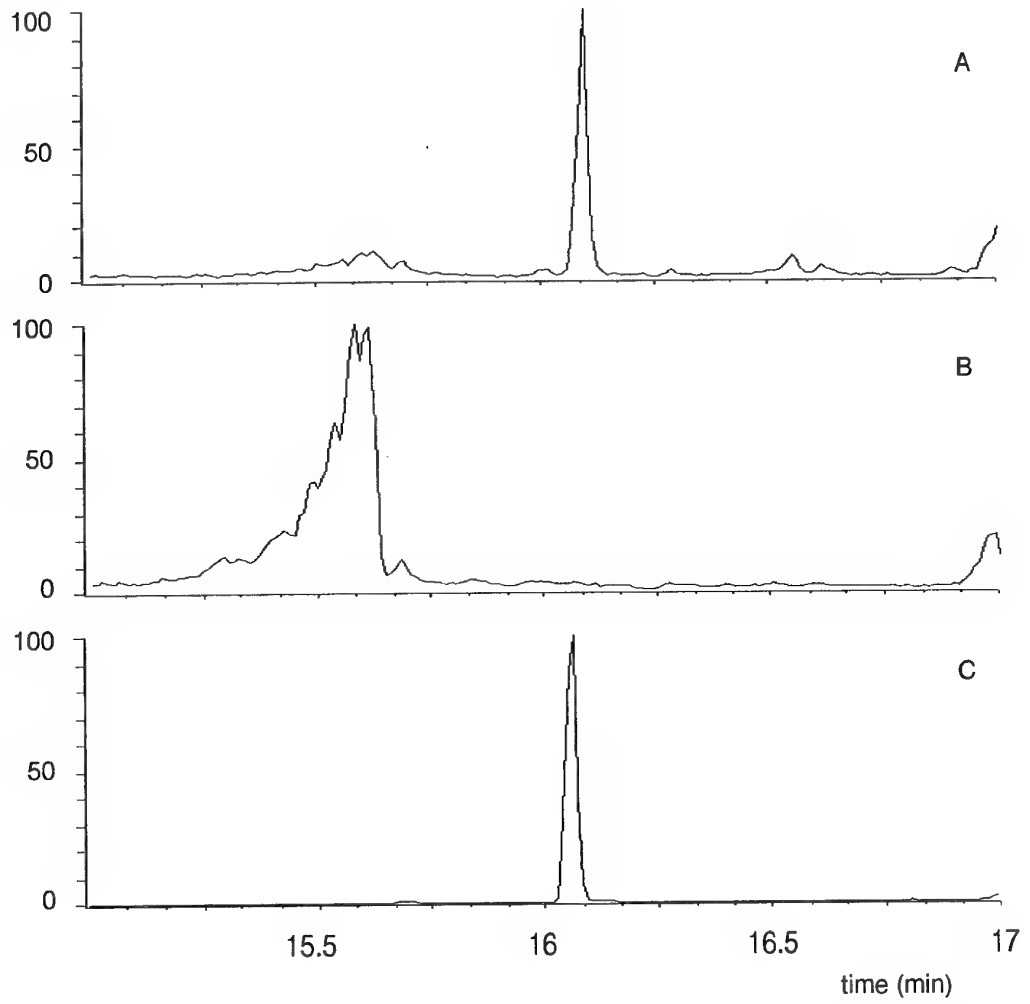


Figure 11. GC-NCI/MS analysis of globin (20 mg) isolated from blood taken from an Iranian victim (# 6 in Table 2) 8-9 days after exposure to sulfur mustard (upper panel) and from non-treated human blood (middle panel), after the orginal modifed Edman degradation procedure. Globin (0.1 mg) isolated from blood treated with 10 mM sulfur mustard-$d_8$ served as an internal standard (lower panel). Ion chromatograms after monitoring for *m/z* 564 (analyte) and 572 (internal standard).

## IV.3 Validation of the two Standard Operating Procedures

### IV.3.1 Introduction

In order to validate the two SOPs, both the intra- and inter-individual variation of the *in vitro* sensitivity of human blood for adduct formation of DNA and hemoglobin with sulfur mustard were determined. In addition, both procedures were used in the same sets of blood samples of hairless guinea pigs at increasing time intervals after i.v. administration of sulfur mustard at two doses in order to demonstrate that the procedures are mutually confirming, to demonstrate that the results depend on the dose, and to obtain data on the persistence of the N7-HETE-Gua in DNA of white blood cells as well as of the N-terminal valine adduct in hemoglobin. Similar persistence studies were performed after i.v. administration of one dose of sulfur mustard to a marmoset. The immunoslotblot assay was also validated for N7-HETE-Gua in DNA of epidermal cells after administration of sulfur mustard to the skin of the hairless guinea pig.

### IV.3.2 Determination of the intra-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard using the immunoslotblot assay for N7-HETE-Gua

The intra-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard was determined in blood samples taken from 5 donors at 3 time points, by using the immunoslotblot assay. The results are summarized in Figure 12. The data indicate a large intra-individual variation after exposure to 5 µM sulfur mustard, particularly for donors 3 and 4. The mean variance at 5 µM calculated as the average of the variances for each donor was 3.2. The variation for donor 3 is much less at 1 µM sulfur mustard exposure, in spite of the lower adduct level, which suggests that the intra-individual variation is not due to variation in sensitivity of the blood for DNA-adduct formation. Rather, these results might be due to irregularities during exposure, DNA isolation or denaturation.

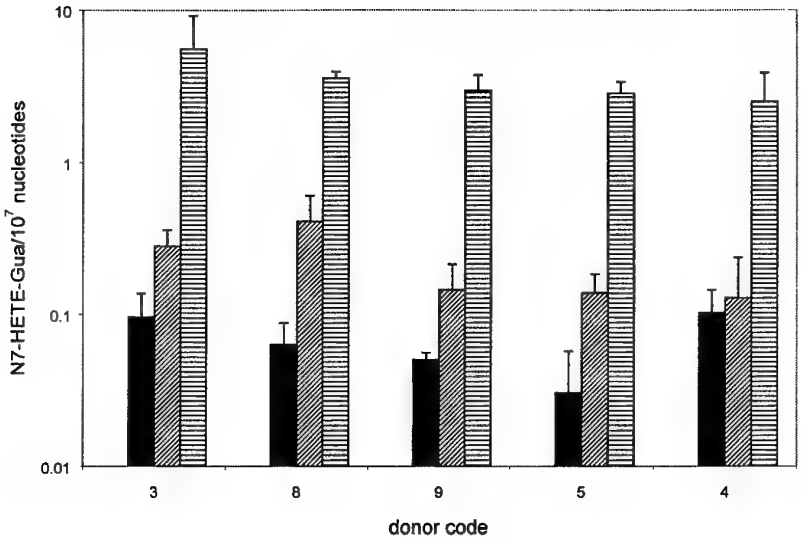


Figure 12. Intra-individual variation for N7-HETE-Gua in DNA in blood exposed to sulfur mustard. On 3 different days blood was collected from 5 donors and exposed to 0, 0.5 μM (filled bars), 1 μM (slashed bars) and 5 μM (bars with horizontal lines) of sulfur mustard for 1 h at 37 °C. After exposure blood samples were stored at -20 °C and analyzed simultaneously in the immunoslotblot assay. The data are ranked to the adduct level after exposure at 5 μM sulfur mustard. No sulfur mustard adducts were observed in unexposed blood samples. The bars represent the mean N7-HETE-Gua level over the three different days. The error bars represent the standard deviation. The mean variance at 5 μM was 3.2.

### IV.3.3 Determination of the intra-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard using GC-MS analysis of N-alkylated terminal valine in hemoglobin

The intra-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard was determined in blood samples taken from 5 individuals at 3 time points, by using the GC-MS analysis of N-alkylated terminal valine in hemoglobin. For this purpose, the blood samples were exposed to sulfur mustard (5 μM), globin was isolated and subjected to the SOP for the modified Edman degradation (see Subsection III.2.8). Only one exposure level was studied in view of the large amounts of samples to be analyzed. The results (see Table 3) indicate that the intra-individual variation is reasonable.

Table 3. Ratios for the peak areas of analyte and internal standard determined in blood samples of five donors taken at three different time points (day 1, day 8, day 15), after *in vitro* exposure to sulfur mustard (5 µM), by using the SOP for determination of alkylated N-terminal valine in hemoglobin. Globin from human blood exposed to sulfur mustard-$d_8$ (10 µM) was used as an internal standard.

| Donor | Peak ratio analyte/internal standard at day 1[a,b] | Peak ratio analyte/internal standard at day 8[a,b] | Peak ratio analyte/internal standard at day 15[a,b] |
|---|---|---|---|
| 1 | 0.35; 0.35 | 0.35; 0.35 | 0.34; 0.20 |
| 2 | 0.37; 0.36 | 0.37; 0.34 | 0.34; 0.38 |
| 3 | 0.34; 0.36 | 0.43; 0.36 | 0.29; 0.30 |
| 4 | 0.34; 0.40 | 0.51; 0.35 | 0.33; 0.29 |
| 5 | 0.47; 0.40 | 0.36; 0.32 | 0.31; 0.30 |

[a] Modified Edman degradations were carried out in duplicate.
[b] Corrected to the use of equal amounts of analyte and internal standard

IV.3.4 Determination of the inter-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard using the immunoslotblot assay for N7-HETE-Gua

The inter-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard was determined in blood samples of 10 donors, by using the immunoslotblot assay for N7-HETE-Gua. Blood samples were exposed to 0, 0.5, 1 and 5 µM of the agent. DNA was isolated in two series, denatured and each DNA sample was analyzed on two different days. In Figure 13 the averages of the four measurements are presented with SEM. To correct for the day-to-day variability, the data are normalized to the average adduct level of the four measurements performed in the blood samples of the 10 donors after exposure to 5 µM sulfur mustard over the average adduct level of the samples determined on the corresponding day. It is clear that the SEMs of the four observations for each blood sample are smaller than the variation in adduct levels in the DNA samples isolated from blood of the various donors. After exposure to 5 µM sulfur mustard the adduct level ranged from 4.4 to 7.2 N7-HETE-Gua/$10^7$ nucleotides. In all unexposed blood samples no N7-HETE-Gua was observed.

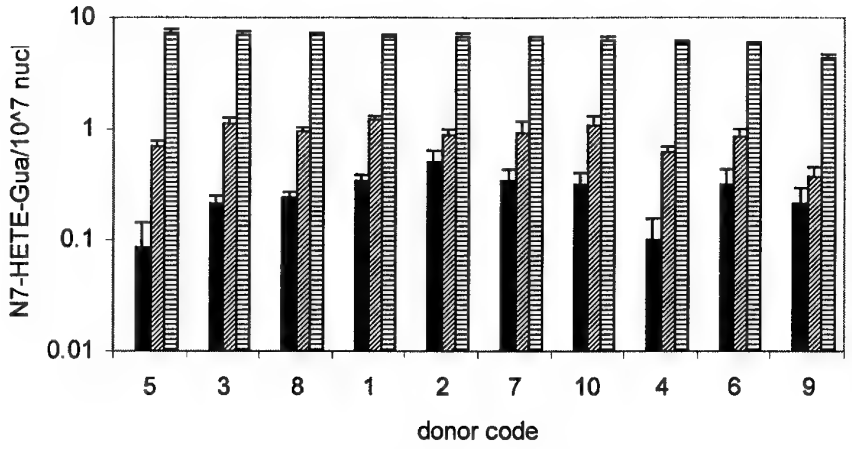


Figure 13. Immunoslotblot assay of N7-HETE-Gua in DNA of human blood of 10 donors exposed *in vitro* to 0, 0.5 µM (filled bars), 1 µM (slashed bars) and 5 µM (bars with horizontal lines) of sulfur mustard for 1 h at 37 °C. DNA was isolated from the blood samples in two series, denatured and each DNA sample analyzed on two different days in the immunoslotblot assay. As correction for the day-to-day variability, the data are normalized to the average adduct level of all four measurements performed in the blood samples of the 10 donors after exposure to 5 µM sulfur mustard over the average adduct level of the samples determined at the corresponding day. The data are ranked to the adduct level after exposure at 5 µM sulfur mustard. In the unexposed blood samples no sulfur mustard adducts were observed. The averages of the four measurements are presented with SEM. The mean variance over the 10 donors amounted to 0.69.

When comparing the inter-individual variation as expressed as the mean variance over the 10 donors, *i.e.*, 0.69, with the mean intra-individual variation (mean variance of 3.2; see Subsection IV.3.2) it can be concluded that the inter-individual variation can be explained fully by the intra-individual variation. The latter might be due to irregularities during exposure, DNA isolation or denaturation.

IV.3.5 Determination of the inter-individual variation of the *in vitro* sensitivity of human blood to sulfur mustard using GC-MS analysis of N-alkylated terminal valine in hemoglobin

The inter-individual variation of *in vitro* sensitivity of human blood to sulfur mustard was determined in blood samples of 10 donors by using the GC-MS analysis of N-terminal alkylated valine in hemoglobin. Only one exposure level was studied in view of the large amounts of samples to be analyzed. For this purpose, the blood samples were exposed to sulfur mustard (5 µM), globin was isolated and subjected to the SOP for the modified Edman degradation (see Subsection III.2.8). The results (see Table 4) indicate that the inter-individual variation is reasonable.

Table 4. Ratios for the peak areas of analyte and internal standard determined in blood samples of 10 donors, after *in vitro* exposure to sulfur mustard (5 µM), by using the SOP for determination of alkylated N-terminal valine in hemoglobin. Globin from human blood exposed to sulfur mustard-$d_8$ (10 µM) was used as an internal standard.

| Donor | peak ratio analyte/internal standard[a,b] |
|---|---|
| 1 | 0.34; 0.20 |
| 2 | 0.33; 0.38 |
| 3 | 0.29; 0.30 |
| 4 | 0.33; 0.29 |
| 5 | 0.31; 0.30 |
| 6 | 0.35; 0.30 |
| 7 | 0.30; 0.28 |
| 8 | 0.36; 0.31 |
| 9 | 0.38; 0.31 |
| 10 | 0.34; 0.23 |

[a] The modified Edman degradation was carried out in duplicate.
[b] Corrected to the use of equal amounts of analyte and internal standard.

IV.3.6 Dose effect relationship for N7-HETE-Gua induction in DNA of white blood cells and for N-alkylated terminal valine induction in hemoglobin after i.v. administration of 0.1 and 0.5 LD50 of sulfur mustard to hairless guinea pigs

It was proposed to determine a dose-effect curve for N7-HETE-Gua induction in DNA of white blood cells and for alkylated N-terminal valine induction in hemoglobin after i.v. administration of 0.02, 0.1 and 0.5 LD50 of sulfur mustard to hairless guinea pigs (LD50 is 8.2 mg/kg for hairless guinea pigs; Langenberg *et al.*, 1998). Since previous experiments in which 0.3 and 1 LD50 (i.v.) of sulfur mustard had been administered (Langenberg *et al.*, 1998), showed some uncertainty about the rate of formation of N7-HETE-Gua adducts in white blood cells, the dose-effect curve was obtained at two time points after administration, *i.e.*, 10 min and 1 h. Furthermore, blood was collected by heart puncture thus avoiding treatment with heparin prior to sulfur mustard administration: in previous experiments insufficient amounts of DNA with low purity were isolated, probably due to coagulation and precipitation observed after thawing of frozen blood samples. After performing the experiment with 0.1 LD50 it was concluded that the adduct levels in the experiment with 0.02 LD50 would be too low to be determined. Consequently, this experiment was skipped.

In all cases, DNA could be isolated from blood albeit with variable purity ($A_{260}/A_{280}$ between 1.4 and 1.7). The adduct level was 2-4 N7-HETE-Gua/$10^7$ nucleotides (see Table 5), *i.e.*, in the same range as observed previously after exposure to 0.3 LD50 of the agent (Langenberg *et al.*, 1998). The adduct level determined 1 h after exposure increased significantly with dose.

Table 5 N7-HETE-Gua (per $10^7$ nucleotides) determined in blood samples of guinea pigs collected at the time points indicated after i.v. administration of sulfur mustard (0.1 or 0.5 LD50).

| Dose of sulfur mustard | N7-HETE-Gua levels in DNA of white blood cells[a] | |
|---|---|---|
| | 10 min after administration | 1 h after administration |
| 0.1 LD50 | 2.5 (1.2) | 1.3 (0.8) |
| 0.5 LD50 | 2.4 (0.4) | 3.9 (0.3) |

[a] Averages of 4-6 determinations with the standard error of the mean (between brackets)

The results obtained for the levels of N-alkylated terminal valine in hemoglobin (see Table 6) clearly demonstrate that these amounts depend on the dose.
In case of an exposure corresponding to 0.5 LD50, both the N7-HETE-Gua and the N-alkylated terminal valine levels are 1 h after exposure significantly higher than after 10 min.

Table 6. Ratios for the peak areas of analyte and internal standard determined in blood samples of hairless guinea pigs after administration of sulfur mustard (0.1 and 0.5 LD50) by using the SOP for determination of alkylated N-terminal valine in hemoglobin. Globin from human blood exposed to sulfur mustard-$d_8$ (10 μM) was used as an internal standard.

| Dose of sulfur mustard | Peak ratio analyte/internal standard[a,b] | |
|---|---|---|
| | 10 min after administration | 1 h after administration |
| 0.1 LD50 | 0.017; 0.014 | 0.018; 0.018 |
| 0.5 LD50 | 0.038; 0.033 | 0.064; 0.058 |

[a] Modified Edman degradations were carried out in duplicate.
[b] Corrected to the use of equal amounts of analyte and internal standard.

IV.3.7 Persistence of N7-HETE-Gua in DNA of epidermal cells after skin exposure of hairless guinea pigs to various Ct values of sulfur mustard vapor

It was found in previous experiments (Langenberg *et al.*, 1998) that isolation of DNA from skin of hairless guinea pigs is cumbersome due to a relatively thick horny layer and a rather thin epidermis (about 2 - 3 cell layers) In order to obtain reliable dose-effect curves as part of the validation of the immunoslotblot assay, the isolation of DNA from skin of the hairless guinea pig was improved by taking more skin tissue (3 times a piece of 5 × 5 mm) and by filtration of the cell suspension of the epidermis with the (thick) horny layer through a nylon gauze after separation of the epidermis from the dermis. This resulted in sufficient amounts of DNA with a reasonable purity ($A_{260}/A_{280}$ between 1.64 and 1.73).

*In vivo* exposure of hairless guinea pigs to a Ct of 1100 mg.min.m$^{-3}$ (at 28 °C on the skin) of sulfur mustard vapor resulted in a marginal adduct level in the skin, *i.e.*, about 0.2 N7-HETE-Gua/$10^7$ nucleotides, which is one order of magnitude lower than that in the skin of a pig ear after *in vitro* exposure to a tenfold lower dose (Van der Schans *et al.*, 1999). These results

indicate that the thick horny layer provides a better protection than that of the pig ear skin at this short-lasting exposure.

A reasonable adduct level was induced after a local exposure with saturated sulfur mustard vapor for 4-8 min (Ct about 4400 – 8800 mg.min.m$^{-3}$). The adduct level amounted to 2.0 N7-HETE-Gua/10$^7$ nucleotides in biopsies collected at 70 min after ending a 4 min exposure, but adduct formation was not yet observed in biopsies collected at 20 min after exposure. After exposure for 8 min, adduct formation was observed already at 30 min after exposure (4.0 N7-HETE-Gua/10$^7$ nucleotides) which had increased still somewhat at 80 min after exposure. Therefore, we decided to carry out the persistence studies after local exposure for 8 min. In order to use less animals, exposures were carried out on a number of locations on the dorsal skin of the animal at various time points before biopsies were collected simultaneously, after euthanasia.

The results are summarized in Figure 14. Unfortunately, the animal destinated for the 7, 14 and 28 days-points died untimely. The data indicate that the adduct level was already maximal at 10 min after the end of an 8 min exposure and does not significantly decrease during the first day. On day 2 and 3 the adduct level had decreased by a factor 2-3. On day 17 and 45 the adduct level was marginal but still detectable. This decrease might be due to repair and/or desquamation of the skin.

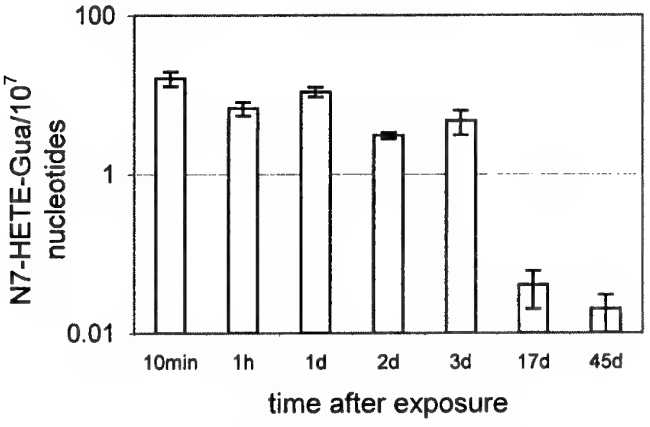


Figure 14. Persistence of N7-HETE-Gua in skin of hairless guinea pig after 8 min *in vivo* exposure to saturated sulfur mustard vapor at 28 °C on the skin, as detected with the immunoslotblot assay. Biopsies were collected at the times indicated. The data represent the mean of two (1, 3, 17 and 45 days) or four determinations (10 min, 1 h and 2 days). The error bars represent the range between the two determinations or the SEM of four determinations, respectively.

### IV.3.8 Persistence of N7-HETE-Gua in blood of the hairless guinea pigs

In the next series of experiments the persistence of both the N7-HETE-Gua adduct and alkylated N-terminal valine in hemoglobin was determined in hairless guinea pigs. To this end, animals were treated with sulfur mustard (0.5 LD50, i.v.) and after 10 min, 1 h, 1 day and 2, 3, 8, 14, 21 and 28 days the animals were killed and blood was collected by heart puncture. The blood samples were divided into two parts: one part for the analysis of the alkylated N-terminal

valine residue in hemoglobin and the other part for the immunoslotblot analysis of N7-HETE-Gua. For each time point of interest, a separate animal was exposed since it is not possible to take more than one blood sample that is sufficiently large to perform both analyses.

The isolation of DNA from blood of the hairless guinea pig again encountered problems which resulted in too low $A_{260}/A_{280}$ ratios. This could be improved by carrying out the protein precipitation step on ice. Nevertheless, sufficient DNA could not be isolated from the blood samples taken 8 or 21 days after sulfur mustard administration.

The results of the immunoslotblot assay presented in Figure 15 indicate that the adduct level was reasonably stable during the first 14 days but adducts disappeared completely during the second period of two weeks.

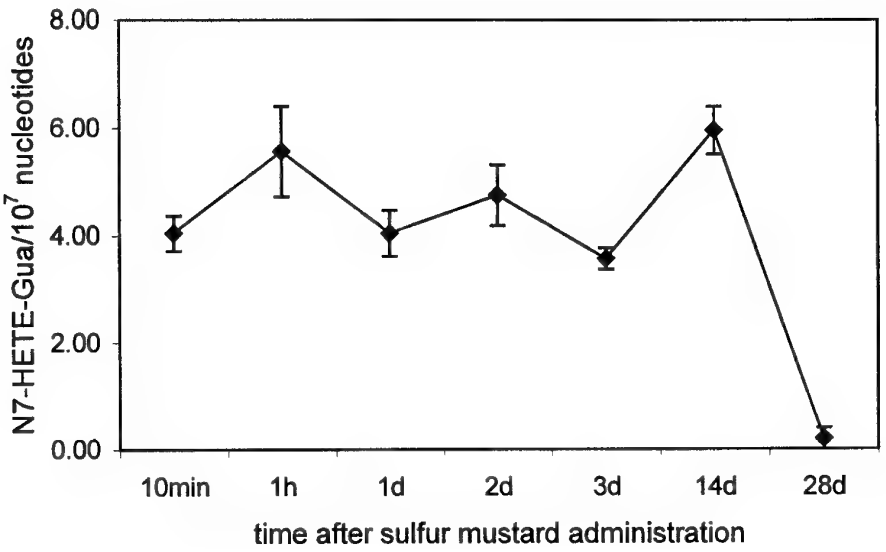


Figure 15. Persistence of N7-HETE-Gua in blood of the hairless guinea pig after sulfur administration (4.1 mg/kg, i.v.; 0.5 LD50). At the time points indicated, blood was collected by heart puncture and treated in small parts (300 µl) with RBC lysis buffer, centrifuged and frozen. Two animals were used for each time point. The averages of 6-17 measurements (after 28 days the average of 3 measurements) are presented. The error bars represent the SEM.

Adduct levels after a dose of 0.1 LD50 were detectable up to at least 1 h after sulfur mustard administration (Table 7). Some blood samples of the persistence experiments performed in animals treated with 0.5 LD50 of the agent were reanalyzed simultaneously in order to enable a direct comparison. The latter data appeared somewhat lower than in the previous series of analyses due to procedure uncertainties but adducts were still detectable up to 14 days after sulfur mustard administration.

Table 7. N7-HETE-Gua (per $10^7$ nucleotides) in blood of hairless guinea pigs collected at the time periods indicated after i.v. administration of sulfur mustard (0.1 or 0.5 LD50).

| Dose | N7-HETE-Gua in DNA of white blood cells at the time points | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10 min | 1 h | 1 d | 2 d | 14 d | 28 d | 56 d |
| 0.1 LD50 | 0.70 | 0.42 | | | | | |
| 0.5 LD50 | 1.50 | 1.84 | 0.87 | 1.15 | 0.30 | 0.01 | 0.00 |

IV.3.9 Persistence of N-alkylated terminal valine in hemoglobin of hairless guinea pigs

In order to investigate the persistence of N-alkylated terminal valine in hemoglobin, the blood samples obtained in the experiment described in Subsection IV.3.8 were processed. Globin was isolated and the modified Edman degradation was performed, followed by derivatization with heptafluorobutyryl imidazole, according to the SOP (see Subsection III.2.8). Globin isolated from human blood exposed to sulfur mustard-$d_8$ (10 µM) was used as an internal standard. The results are summarized in Table 8 and shown in Figure 16. Care has to be taken with the interpretation of the results since the experiments were performed with separate animals for each time point. In globin isolated from blood of a non-exposed hairless guinea pig the adduct could not be detected. The adduct level seems to increase during the first hour of the experiment. Apparently, a considerable amount of free sulfur mustard circulates in the blood of the animal for the first hour, leading to an accumulation of adducts. Between 1 h and 14 days post-administration, the adduct level remains fairly stable. The adduct level clearly decreases in the period between 28 – 56 days after administration. The results show that the adduct is still detectable after 56 days.

Table 8. Ratios for the peak areas of analyte and internal standard determined in blood samples of hairless guinea pigs after administration of sulfur mustard (4.1 mg/kg; 0.5 LD50, i.v.) by using the SOP for determination of alkylated N-terminal valine in hemoglobin. Globin from human blood exposed to sulfur mustard-$d_8$ (10 µM) was used as an internal standard. For each time point a separate hairless guinea pig was exposed.

| Time point | Peak ratio analyte/internal standard[a,b] | mean |
|---|---|---|
| 10 min | 0.0379; 0.0330 | 0.036 |
| 1 h | 0.0578; 0.0637 | 0.061 |
| 1 d | 0.0480; 0.108 | 0.078 |
| 2 d | 0.0640; 0.0666 | 0.065 |
| 3 d | 0.0710; 0.0730 | 0.072 |
| 7 d | 0.0492; 0.0451 | 0.047 |
| 14 d | 0.0551; 0.104[c] | 0.080 |
| 28 d | 0.0494; 0.0519 | 0.051 |
| 56 d | 0.0081; 0.0079 | 0.008 |

[a] Modified Edman degradations were carried out in duplicate, unless stated otherwise.
[b] Corrected to the use of equal amounts of analyte and internal standard.
[c] Determinations performed in two animals; modified Edman degradations were carried out once.

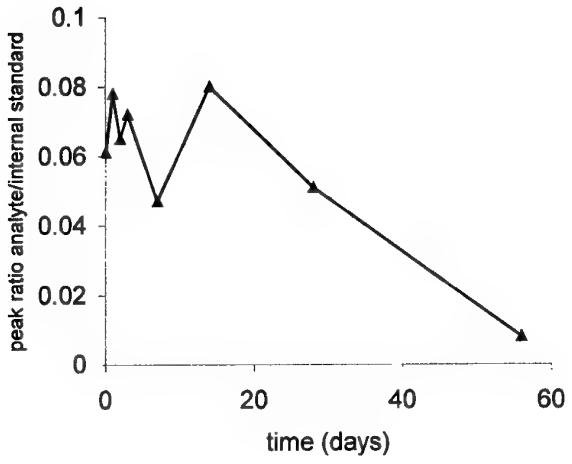


Figure 16. Persistence of alkylated N-terminal valine in hemoglobin of hairless guinea pigs after sulfur mustard administration (4.1 mg/kg; 0.5 LD50, i.v.). At the time points indicated blood was collected (heart puncture), using different animals for each time point. Globin was isolated and analyzed according to the SOP for determination of N-HETE-Val. Globin from human blood exposed to sulfur mustard-$d_8$ (10 μM) was used as an internal standard.

IV.3.10 Persistence of N7-HETE-Gua in blood of the marmoset

Originally, we proposed to study the persistence of N7-HETE-Gua in blood of a marmoset after i.v. administration of 1.5 mg sulfur mustard/kg, *i.e.*, the estimated 0.5 LD50. In the mean time, we learned from parallel experiments (Langenberg *et al.*, 1998) that these animals survived even a dose of 8.2 mg sulfur mustard/kg body weight. Moreover, the level of N7-HETE-Gua in blood of the guinea pig was rather low after administration of 4.1 mg sulfur mustard/kg body weight. Therefore, we decided also to administer a higher amount of sulfur mustard to the marmoset than originally proposed, *i.e.*, 4.1 mg/kg body weight. Part of each blood sample drawn (100-200 μl of a 300 μl sample) was used for analysis of alkylated N-terminal valine in hemoglobin (see Subsection IV.3.11). Too small amounts of DNA were isolated from the residual part. Therefore, the same marmoset was challenged again with the same amount of sulfur mustard at 10 weeks after the first sulfur mustard administration and somewhat larger blood samples (550 μl) were withdrawn. In this way, sufficient DNA could be isolated from blood samples drawn at 1 h, 1 day and 7, 14, 21 and 28 days after sulfur mustard administration and part of each blood sample (100-200 μl) could be used for parallel determination of alkylated N-terminal valine in hemoglobin (see Subsection IV.3.11).

Adduct levels are presented in Table 9. The data indicate that the N7-HETE-Gua level in the marmoset was somewhat less than in the guinea pig to which the same dose of sulfur mustard was administered. Moreover, the N7-HETE-Gua level had decreased to a low level at 1 day after sulfur mustard administration. A marginal level of N7-HETE-Gua was still observed at 7 days after sulfur mustard administration whereas the adduct could not longer be detected at longer periods after exposure.

Table 9. Levels of N7-HETE-Gua in blood of a marmoset determined with the immunoslotblot assay at various time points after administration of sulfur mustard (4.1 mg/kg, i.v.). Data points are the averages of two determinations (difference between brackets).

| Time after administration | N7-HETE-Gua/ $10^7$ nucleotides | Time after administration | N7-HETE-Gua/ $10^7$ nucleotides |
|---|---|---|---|
| 1 h | 1.05 (0.05) | 14 d | 0.00 |
| 1 d | 0.04 (0.005) | 21 d | 0.00 |
| 7 d | 0.03 (0.001) | 28 d | 0.00 |

IV.3.11 Persistence of N-alkylated terminal valine in hemoglobin of marmosets

The persistence of N-alkylated terminal valine in hemoglobin was determined after i.v. administration of sulfur mustard (4.1 mg/kg) to a marmoset. Globin was isolated from blood samples obtained in the parallel experiment for studying the persistence of N7-HETE-Gua. The modified Edman degradation was performed, followed by derivatization with heptafluorobutyryl imidazole, according to the SOP (see Subsection III.2.8). The results are summarized in Table 10 and shown in Figure 17. In the blank sample, the adduct could not be detected. The results suggest that initially the adduct levels increase, as is also the case for the exposed hairless guinea pig. However, since no samples were taken between day 1 and 7, the maximum adduct level cannot be determined. Comparison with the results for the hairless guinea pig shows that adduct levels in marmoset blood are approximately one order of magnitude higher. Roughly the same results were obtained when blood samples were taken from the animal after re-administration of the same dose of sulfur mustard (see Figure 17), although the adduct levels in the second series are slightly lower. In this respect, it has to be remarked that a measurable adduct level should have been present 70 days after the first administration of sulfur mustard (not checked). The results of this series show that the adduct is still detectable after 94 days. Care has to be taken with the interpretation of these results since the adducts were determined in one animal only. Moreover, relatively large blood samples (0.55 ml) were taken up to 28 days after the second challenge. This artificial removal of hemoglobin may lead to a more rapid decrease of the adduct level, which is superimposed on the decrease caused by the decay of the red blood cells.

Table 10. Ratios for the peak areas of analyte and internal standard determined in blood samples of a marmoset after i.v. administration of sulfur mustard (4.1 mg/kg) and after re-administration of the same dose after 70 days, by using the SOP for determination of alkylated N-terminal valine in hemoglobin. Globin from human blood exposed to sulfur mustard-$d_8$ (10 µM) was used as an internal standard.

| Time point | Peak ratio analyte/internal standard[a] | |
|---|---|---|
| | after first challenge | after second challenge |
| 1 h | 0.582 | 0.471 |
| 1 d | 0.707 | 0.486 |
| 7 d | 0.734 | 0.539 |
| 14 d | 0.585 | 0.470 |
| 21 d | 0.459 | 0.364 |
| 28 d | 0.385 | 0.313 |
| 56 d | 0.118 | 0.136 |
| 65 d | | 0.108 |
| 70 d | | 0.078 |
| 78 d | | 0.042 |
| 84 d | | 0.021 |
| 94 d | | 0.004 |

[a] Corrected to the use of equal amounts of analyte and internal standard.

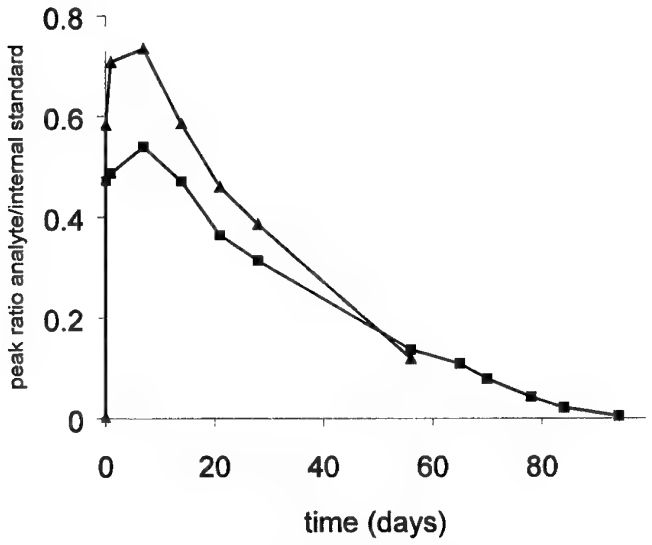


Figure 17. Persistence of alkylated N-terminal valine in hemoglobin of a marmoset after first administration (▲) of sulfur mustard (4.1 mg/kg, i.v.) and after re-administration (■) of the same dose after 70 days. At the time points indicated blood samples were collected, globin was isolated and analyzed by using the SOP for determination of alkylated N-terminal valine. Globin from human blood exposed to sulfur mustard-$d_8$ (10 µM) was used as an internal standard.

### IV.3.12 Applicability of the SOP for immunochemical detection of N7-HETE-Gua in the U.S. Army Medical Research Institute of Chemical Defense (MRICD)

In this section the experiments are described which were performed at MRICD in order to confirm the applicability of the SOP for immunochemical detection of N7-HETE-Gua. In more detail, experiments were performed to demonstrate that the immunoslotblot assay for N7-HETE-Gua in DNA of white blood cells and skin cells can be set up within a short period of time in an independent laboratory by analysing the adduct in (i) calf thymus DNA exposed to sulfur mustard (at TNO-PML), (ii) human blood exposed to sulfur mustard (at TNO-PML) and DNA isolated at MRICD, (iii) human blood exposed to sulfur mustard (at MRICD) and DNA isolated at MRICD, (iv) human skin exposed to sulfur mustard vapor (at TNO-PML) and DNA isolated at MRICD, and (v) human skin of a sulfur mustard victim (USA) and DNA isolated at MRICD.

*Immunoslotblot assay with calf thymus DNA calibration samples*

On day 2, we obtained the first results with the immunoslotblot assay carried out at MRICD according to the SOP, with the exceptions that we used a vortex instead of a shaking device during the processing of the DNA samples on the nitrocellulose filter, another type UV crosslinking source than that used at TNO-PML and another type of luminometer. The results indicated that the assay was performed in a proper way, albeit that there were some irregularities in the first two rows on the nitrocellulose filter. The latter may be due to the improper sealing and fixation of the filter on the microtiter plate. These rows were not used in further experiments to avoid this problem. The dose-effect curve for the calibration samples seems to be similar to that obtained with the same samples at TNO-PML. The results obtained after denaturation with two different formamide batches were not significantly different. In Figure 18 the dose-effect curve obtained with one of the two formamide batches is shown. From the data presented in this figure, it was estimated that the lower detection limit for N7-HETE-Gua is ca. 1 adduct among $1.0 \times 10^8$ unmodified nucleotides, for which a chemiluminescence will be determined corresponding with 3 times the chemiluminescence determined over unexposed DNA.

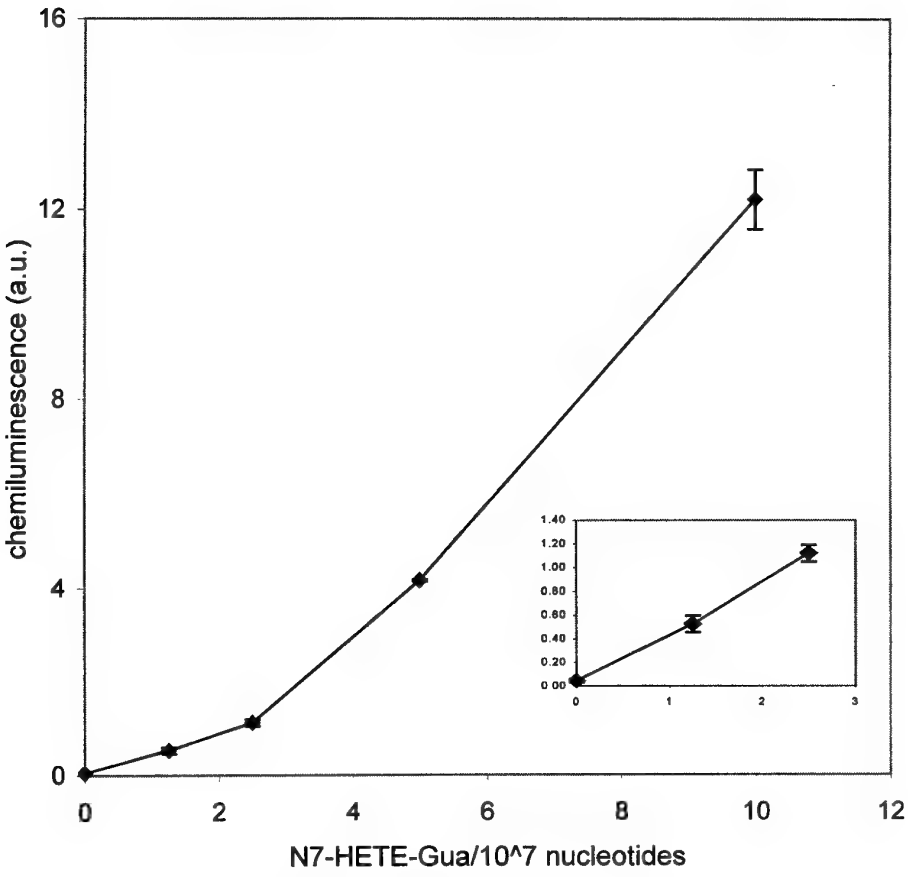


Figure 18 Chemiluminescence in the immunoslotblot assay as a function of the N7-HETE-Gua level in the calf thymus DNA calibration samples exposed to sulfur mustard. Data are the mean of the chemiluminescence of 2 DNA samples. The error bars represent the range between the 2 determinations. Insert: plot at the lower adduct levels.

*DNA isolation from human blood exposed to sulfur mustard (at TNO-PML)*

DNA was isolated from human blood samples exposed to sulfur mustard at TNO-PML by using both the PureGene DNA isolation kit, according to the SOP, and the American analogue, the Masterpure™ Complete DNA and RNA isolation Kit. The American Masterpure™ kit yielded about 70% of the amount of DNA obtained with the PureGene kit. This means that in some cases too small amounts of DNA were available to carry out the immunoslotblot assay twice. The $A_{260}/A_{280}$ ratios determined in the DNA samples revealed that the samples obtained with both kits were sufficiently pure.

*Immunoslotblot assay of the DNA samples isolated from human blood exposed to sulfur mustard*

The results obtained with the immunoslotblot assays indicate that N7-HETE-Gua can be detected in human blood exposed to sulfur mustard at MRICD (Figure 19). Exposure of human blood to 0.5 µM sulfur mustard appeared to be detectable.

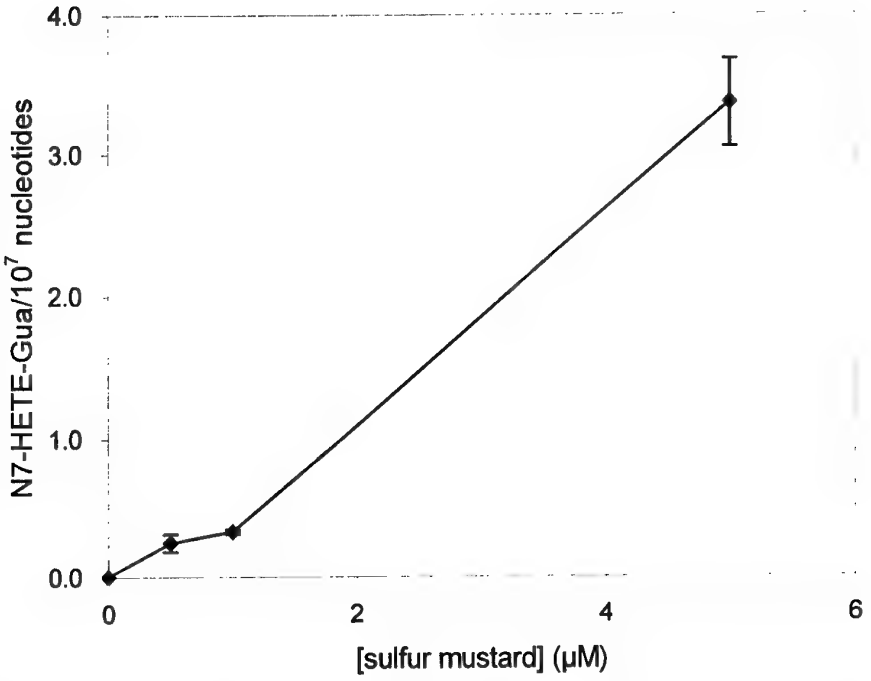


Figure 19. Immunoslotblot assay of N7-HETE-Gua in DNA of blood exposed to sulfur mustard (at TNO-PML). The data represent the average of the adduct levels derived from the chemiluminescence of two samples in relation to that of the calibration DNA samples. The error bars represent the range between those.

N7-HETE-Gua could also be detected in the DNA samples isolated from blood exposed to sulfur mustard at MRICD. However, only estimated adduct levels could be derived due to irregularities in this experiment with the shaking device. These adduct levels appeared to be in the same range as observed for the samples isolated from blood exposed to sulfur mustard at TNO-PML.

*DNA isolation from human skin exposed to sulfur mustard vapor*

Human skin exposed at TNO-PML to sulfur mustard vapor was transferred to MRICD and DNA was isolated. After DNA precipitation, hardly any pellet was observed. Nevertheless, 0.1 TE (50 µl) was added to dissolve the DNA overnight at room temperature. For unknown reasons, only the concentrations of DNA isolated from skin samples which were exposed to saturated sulfur mustard vapor for 0 and 10 s were high enough to allow detection of adducts with the immunoslotblot assay. The DNA isolation procedure on skin samples was repeated resulting this time in reasonable amounts of DNA. These samples were taken to TNO-PML for

immunoslotblot analysis to assess that also in the other skin samples N7-HETE-Gua could be detected. The results indicate that this was indeed the case (Figure 20).

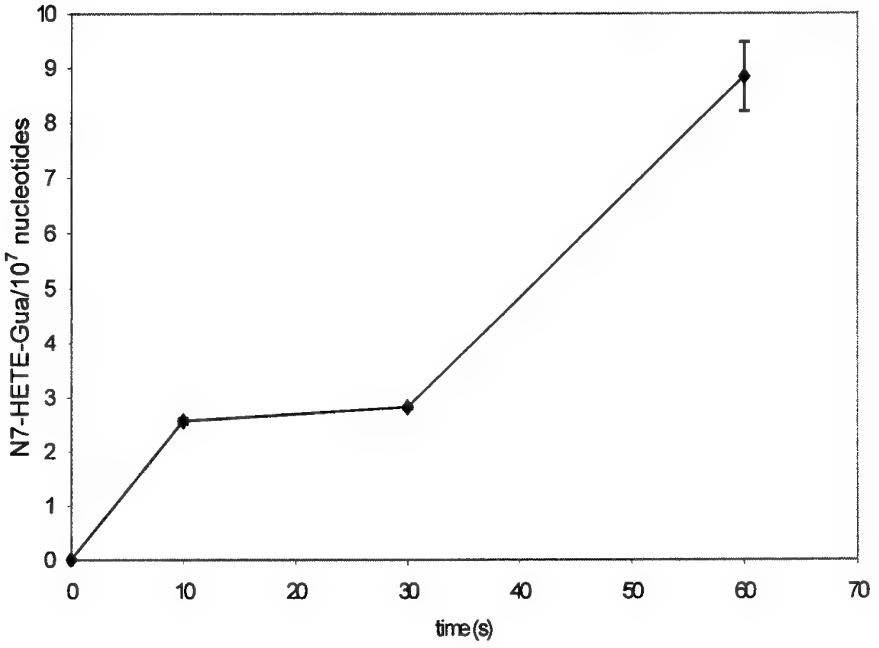


Figure 20. Immunoslotblot assay of DNA of human skin exposed to saturated sulfur mustard vapor (830 $mg.m^{-3}$; 0, 10, 30 and 60 s) at TNO-PML. DNA was isolated at MRICD, which was subsequently processed in the immunoslotblot assay at TNO-PML. The denatured DNA samples were analyzed twice in the immunoslotblot assay. The average of two measurements are presented. The error bars represent the range between those.

*DNA isolation from blister fluid and a piece of the blister of a possible sulfur mustard victim*
The knee of a person who was decontaminating a floor on which some sulfur mustard was spilled was wetted with the decontaminant on the floor. Subsequently, a blister developed on the knee. We attempted to isolate DNA both from a part of the blister fluid and from a piece of the blister. A small amount of DNA (about 1.5 µg) could be isolated from the blister only. After denaturation at a concentration of 25 µg/ml, the DNA solution was mixed (1:1) with denatured calf thymus DNA in order to allow analysis for possible adduct formation in duplicate in the immunoslotblot assay.

*Immunoslotblot assay of the DNA samples isolated from human skin exposed to sulfur mustard and from a blister of a possible sulfur mustard victim*
The DNA samples isolated from human skin and from the blister were analyzed in the immunoslotblot assay. Unfortunately, some irregularities occurred due to problems with shaking (vortex overloaded?) which resulted in much lower chemiluminescence signals than usual and in large differences between duplicate measurements. Therefore, a proper calibration curve could not be derived from this experiment. Nevertheless, adduct formation could clearly be shown for the sulfur mustard-exposed skin sample (Figure 21). Moreover, the sample

isolated from the blister of the possible sulfur mustard-victim also showed an enhanced chemiluminescence.

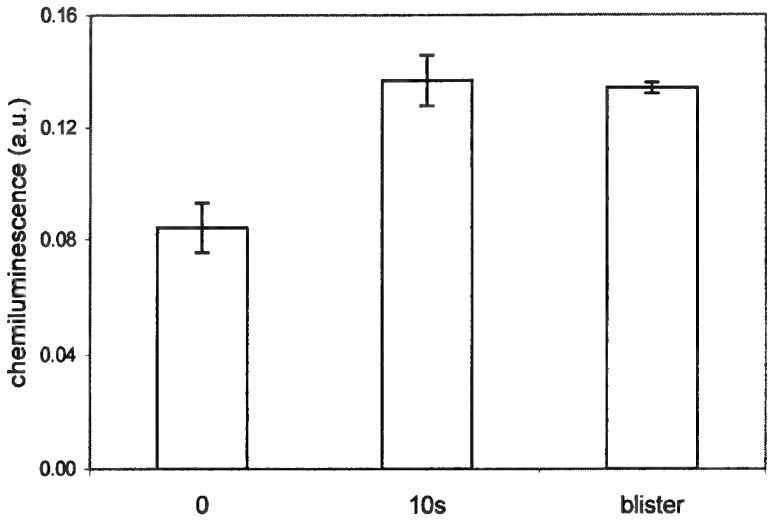


Figure 21. Immunoslotblot assay of N7-HETE-Gua in DNA of human skin exposed to saturated sulfur mustard vapor for 0 and 10 s (Ct of about 140 mg.min.m$^{-3}$) and of DNA isolated from a blister of a possible sulfur mustard victim. The data represent the average of the chemiluminescence of two samples. The error bars represent the range between those. The denatured DNA of a piece of skin from a blister of a possible sulfur mustard victim was mixed 1:1 with denatured calf thymus DNA.

The amount of chemiluminescence detected in the DNA sample isolated from the blister is the same as that in the DNA sample from the 10 s exposed human skin. Since the blister DNA sample was mixed 1:1 with calf thymus DNA, the adduct level in the blister DNA corresponds to that of human skin exposed to saturated sulfur mustard vapor for 20 s. Therefore, an adduct level of 10 N7-HETE-Gua/10$^7$ nucleotides is estimated for the DNA from blistered skin. This is unexpectedly low, because in the case of blister formation much higher adduct levels should be present (Mol, 1992). The reason for the low adduct level might be that the imidazole-ring of most of the N7-HETE-Gua in the skin had become ring-opened due to the (presumed) high pH of the decontaminant applied. The ring-opened structure is not recognized by the antibody.

*Conclusions*

- The immunoslotblot assay for the detection of N7-HETE-Gua in DNA of white blood cells and skin cells could be set up within a half working day after arrival at MRICD and data could be generated within the next one and a half working days. The local technicians were able to perform the assay after a short instruction.
- At the end of day 2, the first immunoslotblot data were available showing proper functioning of the assay.

- The isolation of DNA from the blood samples proceeded as expected. For organizatorial reasons the immunoslotblot assay of these samples was carried out on day 4 and 5.
- A dose-effect curve could be obtained for blood exposed to sulfur mustard.
- N7-HETE-Gua could be detected in a sample of human skin exposed to sulfur mustard vapor.
- A skin sample from a blister of a possible sulfur mustard victim appeared to contain N7-HETE-Gua.

### IV.3.13 Applicability of the SOP for GC-MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin in the U.S. Army Medical Research Institute of Chemical Defense

The applicability of the SOP for the GC-MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin was demonstrated in the MRICD. In more detail, experiments were performed to demonstrate that the SOP can be set up within a short period of time in an independent laboratory by analysing the adduct in (i) globin isolated at TNO-PML from human blood exposed to sulfur mustard and (ii) globin isolated from human blood after exposure to sulfur mustard at MRICD.
Three series of globin samples were analyzed according to the SOP based on GC-NCI/MS analysis after the modified Edman degradation. A survey of the results obtained is given in Table 11. The first series of globin samples were obtained after isolation from human blood that had been exposed to various concentrations of sulfur mustard at TNO-PML. For the second and third series, exposure of human blood to sulfur mustard as well as isolation of globin were performed at MRICD.

The results obtained from the duplicate runs with the first series of globin samples and from the second series of globin samples are presented in Figure 22. A linear dose-effect curve was obtained for these samples. The results obtained from the duplicate runs show the reproducibility of the assay. A lower minimum detectable concentration for exposure of human blood to about 0.1 μM sulfur mustard can be achieved by using this assay, as was derived from the signal-to-noise ratio in ion chromatograms of the GC-NCI/MS analyses.

Results obtained for the third series of globin samples are presented in Figure 23. Results for a part of the globin samples from the first and second series are also given for comparison. The adduct levels in the second series appeared to be ca. 70% of those in the first series. This may be due to a different way of storage of the sulfur mustard stock solutions in acetonitrile or the different conditions during incubation with sulfur mustard, *i.e.*, 2 h at 37 °C for the first series and ca. 4 h at room temperature for the second series. Even lower adduct levels were found in the globin samples isolated from human blood that had been treated with a sulfur mustard solution in saline (third series). These results suggest strongly that sulfur mustard had partly degraded in the saline solution.

Table 11. Ratios of peak areas of analyte and internal standard determined in globin samples isolated from human blood that had been exposed to various concentrations of sulfur mustard, by using the SOP for determination of alkylated N-terminal valine in hemoglobin. Globin from human blood exposed to sulfur mustard-$d_8$ (10 μM) was used as an internal standard.

| Sample[a] | Exposure level (μM) | Peak ratio analyte/internal standard[c] |
|---|---|---|
| 01 | 0 | 0; 0 |
| 02 | 1 | 0.09; 0.08 |
| 03 | 10 | 1.0; 1.0 |
| 04 | 50 | 4.4; 4.7 |
| 05 | 75 | 7.4; 7.1 |
| 06 | 100 | 8.5; 8.8 |
| 07[b] | 5 | 0.42 |
| 08[b] | 10 | 0.83 |
| 09 | 0 | 0 |
| 10 | 1 | 0.04 |
| 11 | 5 | 0.3 |
| 12 | 10 | 0.6 |
| 13 | 100 | 6.2 |
| 14 | 0 | 0; 0 |
| 15 | 0.5 | 0.015; 0.014 |
| 16 | 1.0 | 0.024; 0.026 |
| 17 | 5.0 | 0.12; 012 |

[a] Samples 01-06, 09-13 and 14-17 were globin samples isolated from human blood exposed to sulfur mustard at TNO-PML (first series), globin samples isolated from blood exposed at MRICD to sulfur mustard dissolved in acetonitrile (second series) and globin samples isolated from blood exposed at MRICD to sulfur mustard dissolved in saline (third series), respectively.
[b] Samples 07 and 08 were globin samples isolated from human blood exposed to a mixture of sulfur mustard (5 and 10 μM, respectively) and its deuterated analogue (10 μM).
[c] Corrected to the use of equal amounts of analyte and internal standard.

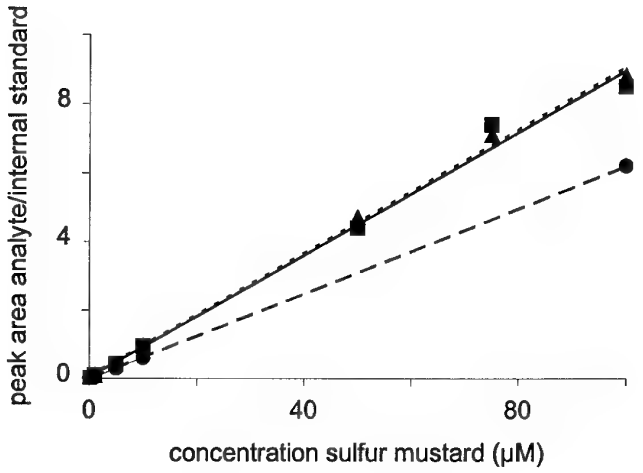


Figure 22. GC-NCI/MS analyses of N-alkylated valine in globin samples isolated from human blood that had been exposed at TNO-PML (■, ▲; duplicate experiments) and at MRICD (●) to various concentrations of sulfur mustard dissolved in acetonitrile and of globin samples isolated from human blood that had been exposed to sulfur mustard-$d_8$ (10 μM), serving as an internal standard, after modified Edman degradation.

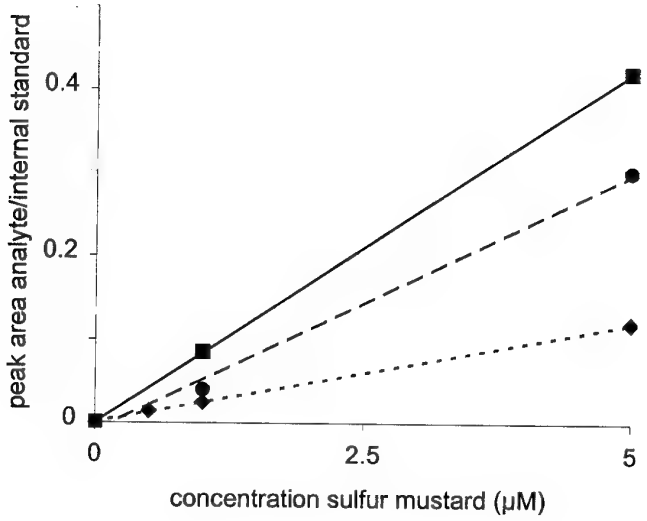


Figure 23. GC-NCI/MS analyses of N-alkylated valine in globin samples isolated from human blood that had been exposed to various concentrations of sulfur mustard dissolved in acetonitrile (■, exposure at TNO-PML; ●, exposure at MRICD) or dissolved in saline (◆, exposure at MRICD), and of globin samples isolated from human blood that had been exposed to sulfur mustard-$d_8$ (10 μM), serving as an internal standard, after modified Edman degradation.

The results show that the SOP for analysis of sulfur mustard adducts to N-terminal valine of hemoglobin could be set up and carried out within a reasonable period of time after arrival at MRICD. On day 3, the first dose-effect curve for human blood exposed to sulfur mustard was available. However, the total procedure can be performed within 9 h, if necessary. The reproducibility and sensitivity of the assay were similar to those achieved at TNO-PML. The adduct levels in hemoglobin of human blood exposed at MRICD using a solution of sulfur mustard in acetonitrile and in saline were about 70% and 35%, respectively, of those in hemoglobin of human blood exposed to sulfur mustard at TNO-PML.

## IV.4 Immunochemical detection of hemoglobin adducts: further exploratory research

### IV.4.1 Introduction

The following steps were taken in our general approach to the development of an immunochemical assay for the detection of sulfur mustard adducts with hemoglobin. It has been attempted to further improve the sensitivity of the immunochemical assay by using the antibodies obtained in the previous agreement (Benschop and Van der Schans, 1995) which were raised against S-HETE-$cys_{93}$ of the β-chain of human hemoglobin. In addition, a synthon derived from adducted N1/N3-histidine was synthesized which was found to be the most abundant adduct formed in hemoglobin after exposure of human blood to sulfur mustard (Benschop and Van der Schans, 1995). Three alkylated peptides representing partial sequences of hemoglobin and containing the adducted histidine were synthesized and served as haptens for raising antibodies. Several clones were selected on their capacity to produce antibodies with specificity for sulfur mustard-treated hemoglobin.

### IV.4.2 Characterization of monoclonal antibodies against cysteine-sulfur mustard adducts in hemoglobin

Several clones of which the antibodies recognized alkylated hemoglobin were obtained from mice immunized with an alkylated peptide, *i.e.*, N-acetyl-S-HETE-$cys_{93}$ through $leu_{106}$-lys of the β-chain of hemoglobin (Benschop and Van der Schans, 1995). One of these, 3H6, was further characterized. These antibodies recognize hemoglobin in a dose-dependent way. It appeared that exposure of human hemoglobin to 50 μM sulfur mustard was detectable in a direct ELISA (Benschop and Van der Schans, 1995). However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have now attempted to apply these antibodies to an immunoslotblot assay for alkylated hemoglobin, but so far without lowering the minimum detectable concentration.

### IV.4.3 Synthesis of peptide haptens containing a histidine-sulfur mustard adduct

During the previous grant it was found that N1/N3-HETE-histidine is the most abundant adduct formed in hemoglobin after exposure of human blood to sulfur mustard. Three specific histidine residues were identified that are alkylated by sulfur mustard, *i.e.*, α-$his_{20}$, β-$his_{77}$, and β-$his_{97}$. We here describe the synthesis of peptide haptens, derived from human hemoglobin, containing these alkylated residues. The required building block was synthesized starting from Nα-Boc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine methyl ester, the synthesis of which was described in the final report of grant DAMD17-92-V-2005 (Benschop and Van der Schans, 1995). The Boc group was selectively removed under the agency of dry HCl (1 M) in ethyl acetate. Subsequently, the ester function was saponified in methanol/water containing 0.2 M

NaOH and finally the Fmoc group was introduced according to a published procedure, affording Nα-Fmoc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine in 70% yield.

The following peptides were synthesized:

1. A-F-S-D-G-L-A-(N1/N3-HETE)H-L-D-N-L-K, which represents the amino acid residues 70-82 of human β-globin
2. G-K-V-G-A-(N1/N3-HETE)H-A-G-E-Y-G-A-K, which represents the amino aicd residues 15-26 (+ lysine) of human α-globin
3. L-(N1/N3-HETE)H-V-D-P-E-N-F-R-L-L-G-N-V-K, which represents the a.a residues 96-109 (+ lysine) of human β-globin.

FPLC analysis showed the presence of one main product in each case. Electrospray MS analysis showed the presence of the expected mass and the sequence of the peptides was firmly established by means of tandem MS analysis. The corresponding native sequences were also synthesized and were used as reference compounds in immunochemical experiments with antibodies raised against the three N1/N3-HETE-histidine-containing peptides.

IV.4.4 Antibodies against peptide haptens containing a histidine-sulfur mustard adduct

We immunized mice with the three different peptide haptens containing a histidine-sulfur mustard adduct, described in Subsection IV.4.3. Subsequently, these mice were used for fusion experiments with the following preliminary results. Clones were selected on their capacity to produce antibodies with specificity for hemoglobin treated with 50 μM sulfur mustard. With hapten 1 two clones were obtained. With hapten 2 one clone was selected and with hapten 3 five clones after subcloning of one clone. Antibodies of these clones (except the one obtained with hapten 3) were tested for specificity on hemoglobin treated with 50 μM sulfur mustard and on keratin treated with 50 or 500 μM sulfur mustard (Table 12). These antibodies show specificity not only for alkylated hemoglobin but also for alkylated keratin. In one case, *i.e.*, clone 190-2H12, the specificity for alkylated keratin seemed to be even higher than for alkylated hemoglobin. This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

In this experiment, the control clone, 3H6, appeared to be negative which suggests that the test system, particularly the coating of the microtiter plates, was still not optimal. Nevertheless, several other clones were positive, suggesting that these clones produced antibodies which were more specific than those of 3H6. During selection of the clones the direct ELISA appeared to be not sufficiently reproducible.

Table 12. Antibody specificities of clones obtained from a fusion after immunization with three peptide haptens containing a histidine-sulfur mustard adduct[a]. Supernatants of cultures were assayed in a direct ELISA on keratin or hemoglobin treated with sulfur mustard

| Clone[a] | Fluorescence intensity[b] observed for | | |
|---|---|---|---|
| | Hemoglobin treated with | Keratin treated with | |
| | 50 μM sulfur mustard | 50 μM sulfur mustard | 500 μM sulfur mustard |
| 183-5B7 | ++ | + | + |
| 183-3D5 | + | - | + |
| 186-1A4 | ++ | + | + |
| 190-4A3 | + | ± | ++ |
| 190-4F5 | ± | - | + |
| 190-2H12 | - | ± | + |
| 190-5E7 | + | - | - |
| 3H6 (control) | - | ± | - |

[a] Clones 183, 186 and 190 were obtained from immunuzation with hapten 1, 2 and 3, respectively, as described in Subsection IV.4.3.

[b] Fluorescence significantly higher than that obtained on the non-alkylated protein is indicated, in increasing order, by ±, +, ++.

## IV.5 Mass spectrometric and immunochemical detection of albumin adducts: exploratory research

### IV.5.1 Introduction

In order to develop an immunochemical assay for the detection of sulfur mustard adducts with albumin, *i.e.*, the most abundant protein in plasma, the following steps were taken in our general approach: quantitation of the binding of the agent to the protein by using [$^{14}$C]sulfur mustard and analysis of tryptic digests of albumin that was exposed to sulfur mustard, for identification of alkylation sites in the protein. One of the alkylated peptides, *i.e.*, the fragment T5 containing an alkylated cysteine, could sensitively be detected in the tryptic digest with LC-tandem MS analysis. Therefore, this alkylated peptide was synthesized and served as a hapten for raising antibodies. Attempts to further decrease the mass spectrometric detection limit for *in vitro* exposure of human blood from analysis of the alkylated T5 fragment (*i.e.* 1 μM) were not successful. Therefore, alternative methods were evaluated for analysis of the alkylated cysteine-34 residue. After pronase treatment of albumin, a small adducted peptide, (S-HETE)Cys-Pro-Phe, *i.e.*, a partial sequence from the T5 fragment, could be sensitively analyzed, allowing a minimum detectable concentration for *in vitro* exposure of human blood of 10 nM. Furthermore, the feasibility was studied of the modified Edman degradation of alkylated aspartic acid as a tool for determination of the possibly formed adduct to the N-terminal amino acid in albumin.

### IV.5.2 Quantitation of binding

Two methods for isolation of albumin from whole blood were examined. The first method (Skipper *et al.*, 1985; Cho *et al.*, 1994), employing ammonium sulfate precipitation, was quite laborious. The second method (Bechtold *et al.*, 1992), based on successive precipitations of

fibrinogen, globulins, and albumin, was more convenient. The purity of the albumin isolated was assessed by SDS-PAGE; both methods gave albumin of high purity (95%).

For quantitation of sulfur mustard binding to the protein, blood was exposed to 1300, 130, 13 and 1.3 μM of $^{14}$C-labeled agent (2 h at 37 °C). After isolation of albumin, the protein (2 mg) was dissolved in a solution of 1 M urea in 0.9% NaCl and radioactivity was determined with liquid scintillation counting. As the specific activity of [$^{14}$C]sulfur mustard (15 mCi/mmol) and the molecular weight of albumin (66.5 kDa) are known, the amount of radioactive material covalently bound per mole of protein could be calculated from the results. A survey of the results is given in Table 13. The results are in the same range as the binding data obtained for hemoglobin

Table 13. Binding of [$^{14}$C]sulfur mustard to human serum albumin upon treatment of human blood with various concentrations of the agent.

| Concentration [$^{14}$C]sulfur mustard (μM) | [$^{14}$C]sulfur mustard bound to albumin per ml blood[a] (nmol) | μmol [$^{14}$C]sulfur mustard bound per 1000 μmol of albumin |
|---|---|---|
| 1.3 | 0.27 (21) | 0.43 |
| 13 | 2.6 (20) | 4.1 |
| 130 | 26 (20) | 41 |
| 1300 | 230 (18) | 370 |

[a] Data within parentheses denote the percentages of total radioactivity added to blood that was bound to albumin

IV.5.3 Identification of alkylation sites for sulfur mustard in albumin

In a first attempt to identify amino acids which are alkylated in albumin by sulfur mustard the protein isolated from blood which had been exposed to [$^{14}$C]sulfur mustard (1 mM) was treated with 6 N HCl. After subsequent derivatization of the amino acid mixture with Fmoc-Cl, two main peaks were present in the HPLC chromatogram (Figure 24). Peak 1 is probably [$^{14}$C]thiodiglycol, resulting from hydrolysis of adducts of glutamic or aspartic acid. Peak 2, which contains 28% of total radioactivity bound to the protein, coelutes with the Fmoc derivative of synthetic histidine-sulfur mustard adduct, *i.e.* Nα-Fmoc-(N1/N3-HETE)histidine. It was not attempted to work out a procedure for determination of exposure to sulfur mustard based on the histidine adduct formed in albumin since up to now this adduct cannot be analyzed by GC-MS or LC-MS at trace levels (Benschop and Van der Schans, 1995).

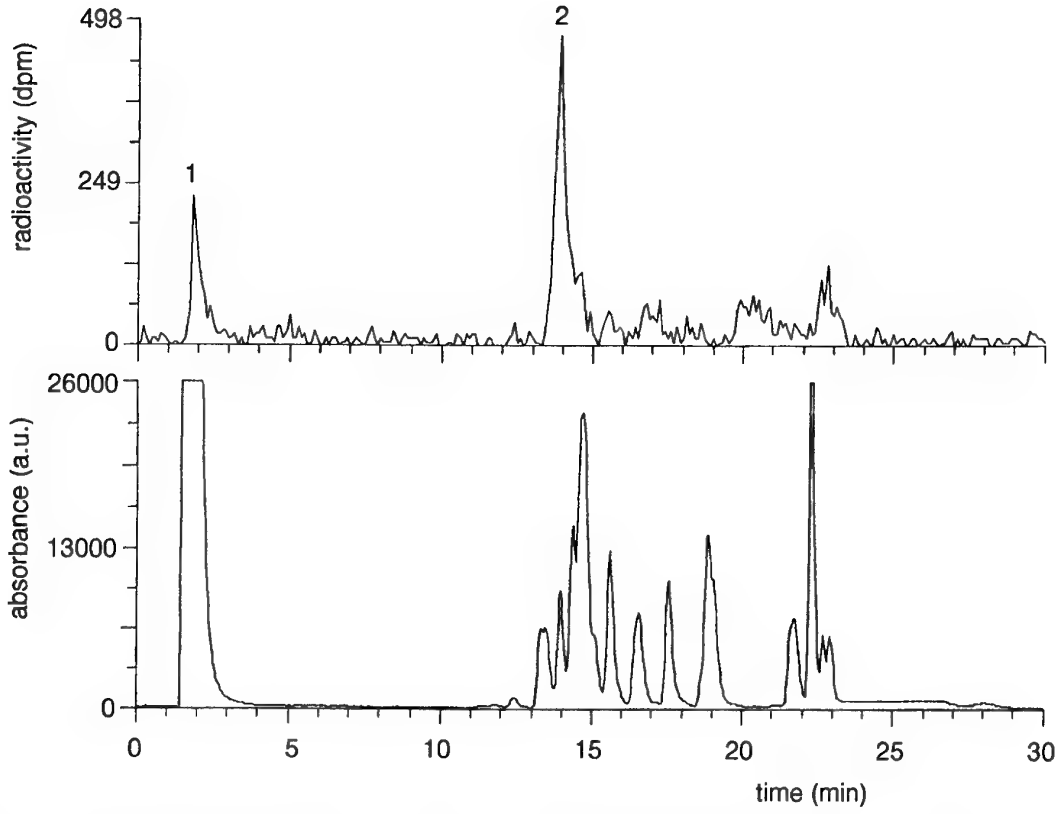


Figure 24. HPLC analysis (PepRPC 5/5 column) of an acidic hydrolysate of albumin isolated from human blood that was exposed to 1 mM $^{14}$C-sulfur mustard, after derivatization with Fmoc-Cl. Upper panel, detection of radioactivity; lower panel, UV detection (254 nm). Eluent (flow 1 ml/min): 0.1% trifluoroacetic acid with a linear gradient to acetonitrile/water/trifluoroacetic acid 48/52/0.1 (v/v/v) in 20 min; peak 1, probably thiodiglycol; peak 2, Nα-Fmoc-N1/N3-HETE-histidine.

In the final report of our previous grant (Benschop and Van der Schans, 1995) we described the use of advanced mass spectrometric techniques for analysis of hemoglobin alkylated by sulfur mustard. We identified several sites of alkylation by sulfur mustard within the tertiary structure of hemoglobin after tryptic digestion of the adducted protein, using tandem mass spectrometry combined with micro-LC. Therefore, we also investigated the feasibility of tandem mass spectrometry to identify alkylation sites for sulfur mustard in albumin after tryptic digestion.

In order to obtain efficient digestion of albumin, the disulfide bridges present in the protein were reduced with dithiothreitol and the resulting free cysteine residues were alkylated with iodoacetic acid. Subsequently, the protein was digested with trypsin. HPLC analysis of tryptic digests of albumin isolated from human blood that was treated with sulfur mustard gave reproducible chromatograms. When albumin was used which was isolated from blood that had been treated with [$^{14}$C]sulfur mustard, a large number of radioactive peaks was observed, demonstrating efficient, albeit dispersed, alkylation of albumin by sulfur mustard. The large peak in the early region of the chromatogram probably represents [$^{14}$C]thiodiglycol (cleavage of

ester adducts) and small alkylated peptides. Fortunately, one peak in the late-eluting region of the chromatogram, containing 4-5% of the total radioactivity bound to the protein, was fully separated from other peptide material (Figure 25). At higher exposure levels an additional peak in the UV region could be observed which coincided with the radioactive peak.

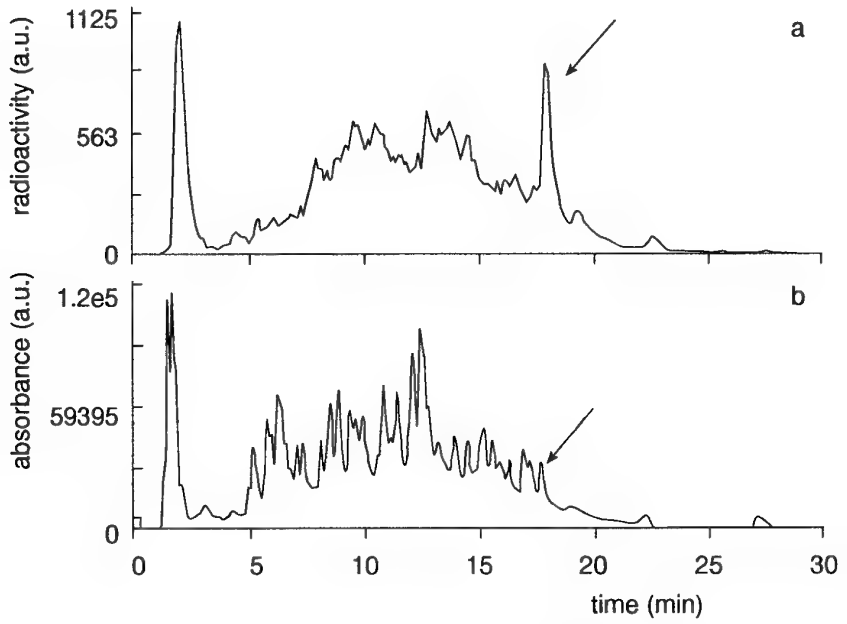


Figure 25. HPLC chromatogram (PepRPC 5/5 column) of a tryptic digest of albumin isolated from human blood that was treated with [$^{14}$C]sulfur mustard (10 mM). A, detection of radioactivity; B, UV detection (214 nm). Eluent: 0.1% TFA in water with a linear gradient to 0.1% TFA in acetonitrile/water (48/52, v/v) in 20 min. The arrow indicates the peak for the alkylated T5 fragment.

Since this peptide represented a relatively high percentage of the total radioactivity bound to albumin and was fully separated from other peptides, our attention was focussed on the identification of this compound. Mass spectrometric analysis of a tryptic digest of albumin from blood exposed to 10 mM sulfur mustard (Figure 26) showed the presence of a compound with *m/z* 1269.3, which corresponds with [$MH_2^{2+}$] of the alkylated T5 fragment, *i.e.*, HETE-(A-L-V-L-I-A-F-A-Q-Y-L-Q-Q-C-P-F-E-D-H-V-K) ($MW_{monoisotopic}$ 2536 Da, $MW_{average}$ 2538 Da).

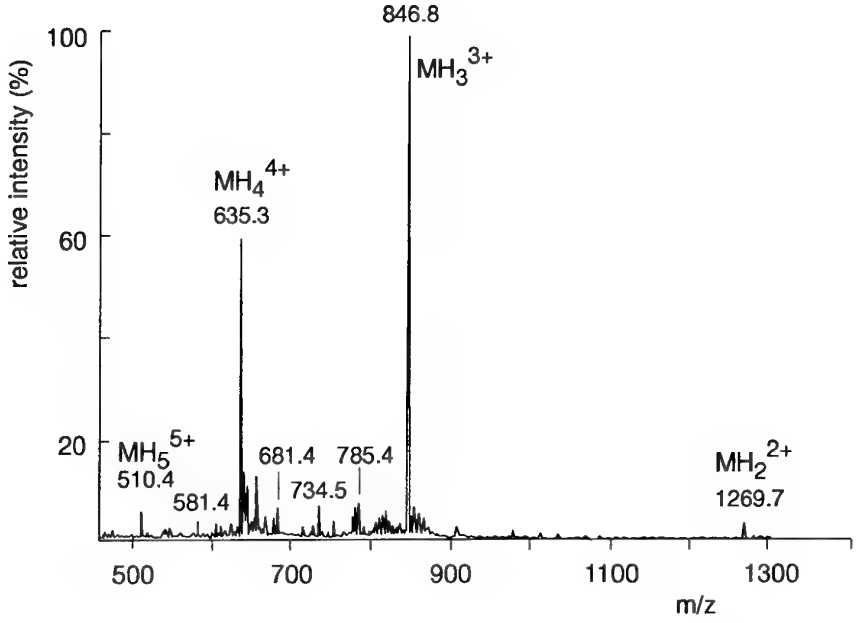


Figure 26. Mass spectrum upon electrospray LC-MS analysis of alkylated T5 peptide in a tryptic digest of albumin isolated from human blood that was exposed to 10 mM sulfur mustard.

The T5 fragment contains the only free cysteine residue of the protein (at position 34) which is believed to be highly reactive towards electrophiles (Bechtold *et al.*, 1992). Tandem MS experiments showed that alkylation had indeed occurred at cysteine-34, which is clearly demonstrated from the *m/z* values of the fragments $Y''_7$ and $Y''_8$ corresponding to values for a nonalkylated and an alkylated fragment, respectively (Figure 27). Moreover, the radioactive peak of the peptide in the tryptic digest coeluted with synthetic T5 alkylated with sulfur mustard at the cysteine, which was readily available by solid phase synthesis. Previously (Benschop and Van der Schans, 1995), peptides containing a cysteine-sulfur mustard adduct were synthesized employing a building block in which the HETE group was protected with a *tert*-butyl group. We now found that the hydroxyl function can be left unprotected (at least for this particular sequence), *i.e.*, employing N-Fmoc-S-HETE-cysteine as a building block for solid phase peptide synthesis. The resulting crude product consisted mainly of the desired S-alkylated T5 and was used without further purification.

The synthetic alkylated peptide served as a hapten for raising antibodies against albumin that has been exposed to sulfur mustard. It also seems worthwhile to investigate whether LC-tandem MS analysis of this peptide in a tryptic digest of albumin is suitable for retrospective detection of exposure to sulfur mustard. As a first step, the detection limit of LC-tandem MS analysis was determined for the synthetic compound. The detection limit of selective ion recording (SIR) for *m/z* 1269.5 ($MH_2^{2+}$) was 10 pg of the alkylated peptide. In the MRM mode the following transitions were recorded:

*m/z* 846.3 ($MH_3^{3+}$) → *m/z* 1071.0, 1014.5, and 978.5
*m/z* 846.3 ($MH_3^{3+}$) → *m/z* 185.0, 284.2, and 397.3

*m/z* 1269.5 ($MH_2^{2+}$) → *m/z* 185.0, 284.2, and 397.3

The most sensitive analysis was obtained from combined recording of the first three transitions, *i.e.*, *m/z* 846.3 → *m/z* 1071.0, 1014.5 and 978.5, allowing a detection of ≥ 15 pg of the alkylated peptide.

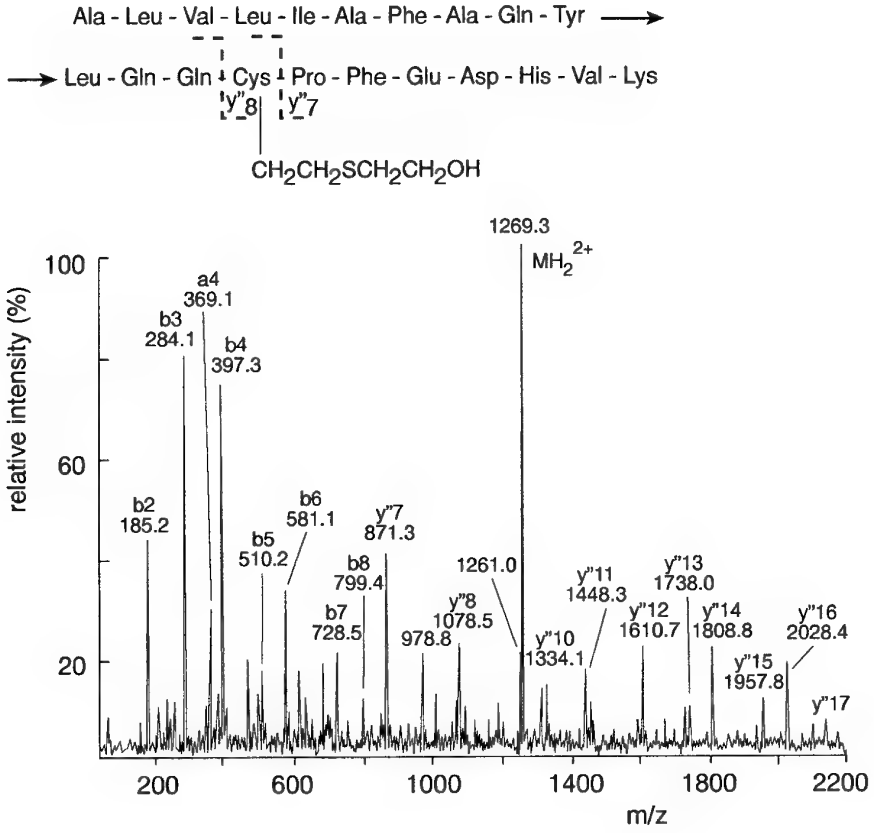


Figure 27. Tandem MS spectrum for molecular ion $MH_2^{2+}$ (*m/z* 1269, see also Figure 26) of alkylated T5 peptide in a tryptic digest of albumin isolated from human blood that was exposed to 10 mM sulfur mustard.

For analysis of trypsinized albumin samples, MRM with high resolution was the method of choice since SIR was not specific enough. The detection limit for the adduct was now increased to 45 pg (from standard addition, S/R 3:1). The minimum detectable concentration for *in vitro* exposure of human blood was determined to be 1 μM (Figure 28). Unfortunately, serious problems were encountered with blank samples, since small signals (just above the detection limit for alkylated T5) were observed at the same retention time as the alkylated T5 fragment. Since impurities in the iodoacetic acid might be responsible for the interferences in the blank, the reduction and alkylation of the isolated albumin were omitted. Unfortunately, the detection limit for the alkylated T5 fragment deteriorated severely because of peak broadening. Some of the analyses were performed in the U.K. using a Q-Tof-MS. However, the detection limit could not be improved due to interfering small signals still present in blank samples.

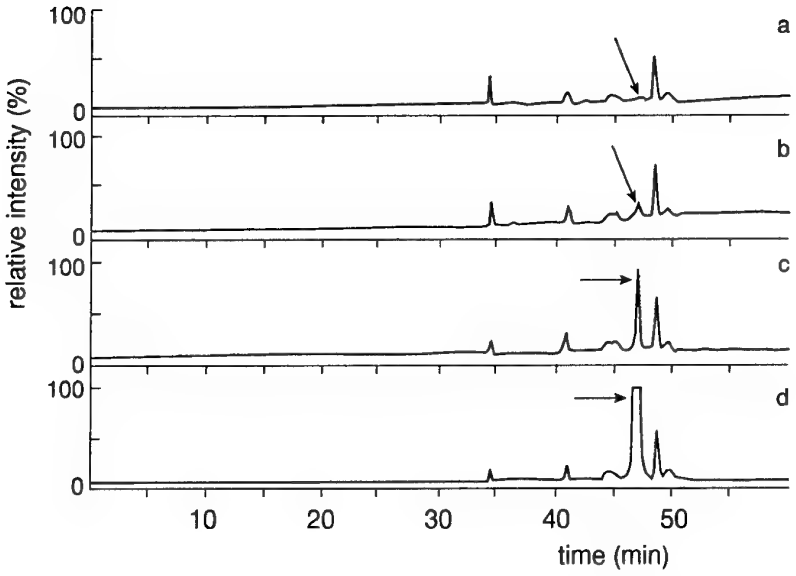


Figure 28. LC-tandem MS analysis of T5 peptide (arrow) in a tryptic digest of albumin, using the multiple reaction monitoring scanning mode for the transition *m/z* 846 ($MH_3^{3+}$) → 1071. Albumin was isolated from non-exposed human blood (A) and from human blood that was exposed to 1 μM (B), 10 μM (C), and 100 μM (D) of sulfur mustard.

We reasoned that a selective modification of the adducted T5 peptide might circumvent the problem that no blank chromatogram could be obtained for an albumin sample isolated from non-exposed blood. From earlier work (Noort *et al.*, 1996b) we learned that treatment of a phosphoserine-thiodiglycol derivative with $H_2O_2$ in a mixture of acetonitrile/acetic acid/water resulted in the rapid formation of the corresponding sulfoxide. Analogous treatment of the sulfur mustard adduct of T5 resulted in the formation of a single product, according to HPLC analysis, with a slightly shorter retention time. LC-tandem-MS analysis showed that this product was the T5 adduct with two sulfoxide functions and MW 2569.9 Da (see Figure 29). Unfortunately, the transition of $MH_3^{3+}$ into doubly charged Y" fragments could be determined less sensitively than in case of the non-oxidized peptide. Moreover, the oxidized peptide could not be detected in tryptic digests treated with $H_2O_2$, which were shown to contain the T5 adduct prior to this treatment. Probably, the oxidized peptide coincided with one of the other peptides in the digest.

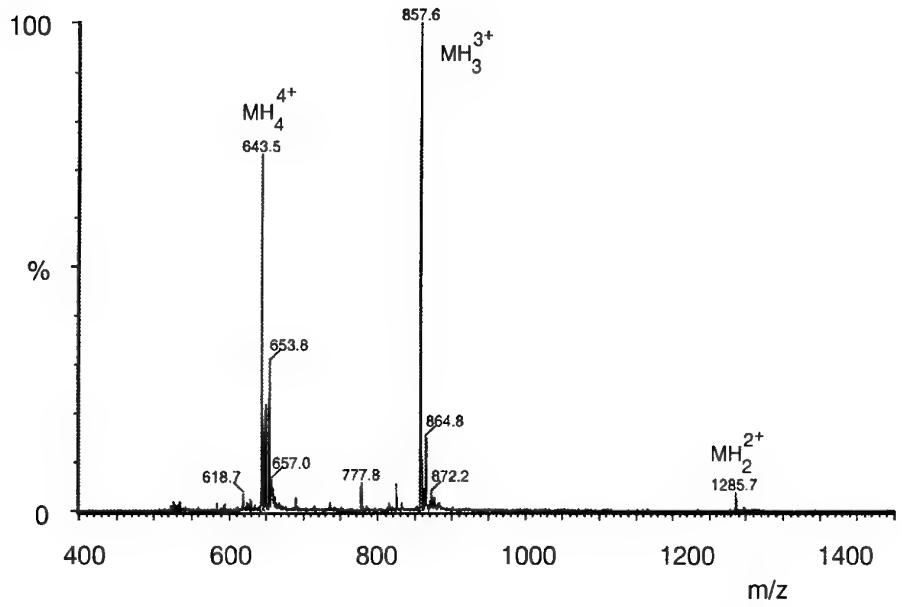


Figure 29. LC-tandem MS spectrum of the oxidized product of the T5 fragment of human albumin containing two sulfoxide functions; $MW_{average}$, 2569.9 Da.

IV.5.4 Assay for detection of sulfur mustard adduct to Cys-34 in albumin

It was attempted to hydrolyze the alkylated T5 fragment with pronase, in order to enable, after derivatization, GC-MS analysis of the adducted cysteine residue. After removal of the enzyme using a filter with a cut-off of 10 kDa, analysis of the incubation mixture with LC-MS did not show the presence of the single adducted amino acid. Instead several small peptides were present, containing the alkylated cysteine residue, *i.e.*, (S-HETE)Cys-Pro (MW 322 Da), (S-HETE)Cys-Pro-Phe (MW 469 Da) and Gln-(S-HETE)Cys-Pro-Phe (MW 597 Da). The tripeptide, which was the most abundant one, could sensitively be detected with LC-tandem MS under MRM conditions: transition *m/z* 470 ($MH^+$) → 105 ($HOCH_2CH_2SCH_2CH_2^+$). The tetrapeptide could be determined by measuring the transition *m/z* 598.2 ($MH^+$) → 263.2. These peptides could also be detected after pronase hydrolysis of a tryptic digest from sulfur mustard-exposed albumin (after reduction and carboxymethylation). A clean blank was obtained when albumin isolated from non-exposed blood was used.

In order to facilitate the procedure, we investigated whether these peptides could be detected after direct digestion with pronase of albumin, which had not been reduced, carboxymethylated and trypsinized. Indeed, the tetrapeptide could be detected in these samples and allowed detection of an exposure level of 10 μM sulfur mustard (see Figure 30). In contrast, a minimum detectable concentration of 0.1 μM was obtained when the tripeptide was analyzed.

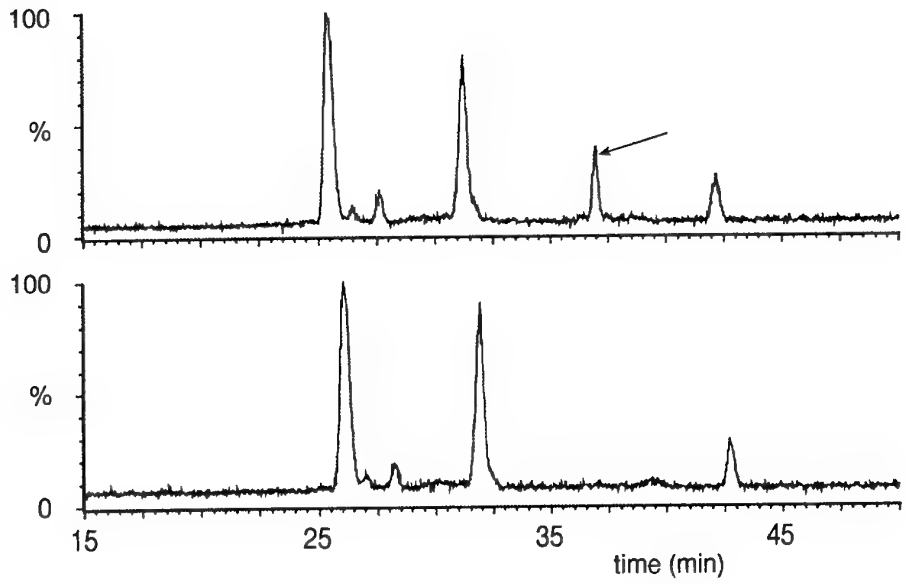


Figure 30. LC-tandem MS analysis of Gln-(S-HETE)Cys-Pro-Phe (arrow) in a pronase digest of albumin, using the multiple reaction monitoring scanning mode for the transition *m/z* 598.2 ($MH^+$) → 263.2. Albumin was isolated from human blood that was exposed to 10 μM of sulfur mustard (upper panel) and from non-exposed human blood (lower panel).

Next, LC-MS analyses with selective ion recording were performed in the pronase digest of albumin isolated from human blood that had been exposed to a relatively high concentration of sulfur mustard (5 mM), in order to compare the relative quantities of the three peptides in the digest. The alkylated di- and tripeptide could be detected. The peak area of the dipeptide was ca. ¾ of that for the tripeptide; the alkylated tetrapeptide was not present in a significant amount. The polar dipeptide eluted as a broad peak together with a number of other peptides. In contrast, the tripeptide eluted as a sharp peak. Therefore, the dipeptide is not suitable as a marker to analyse exposure of albumin to sulfur mustard. For quantitation of the amount of tripeptide formed after pronase digestion, experiments were carried out with albumin isolated from blood which had been exposed to [$^{14}$C]sulfur mustard (1 mM). Upon HPLC analysis of a pronase digest, a small peak was observed which coeluted with the synthetic tripeptide (*vide supra*) and which contained ca. 6% of the total radioactivity bound to the protein. Since a dipeptide containing alkylated cysteine-34 is also formed upon tryptic digestion of alkylated albumin in a considerable amount relative to the tripeptide (ca. ¾, *vide supra*), it is concluded that ca. 10% of the total adducts formed in albumin by exposure to sulfur mustard pertain to alkylated cysteine-34. The somewhat lower percentage determined for the alkylated T5 fragment after tryptic cleavage (4-5%; see Subsection IV.5.3) is probably due to incomplete digestion.

The synthetic tripeptide was obtained by coupling of Nα-Fmoc-(S-HETE)cysteine to the immobilized dipeptide H-Pro-Phe-OH. After deprotection and splitting off from the resin, the tripeptide was isolated as an oil; $^1$H NMR and mass spectrometric data (see Figure 31) were in accordance with the proposed structure.

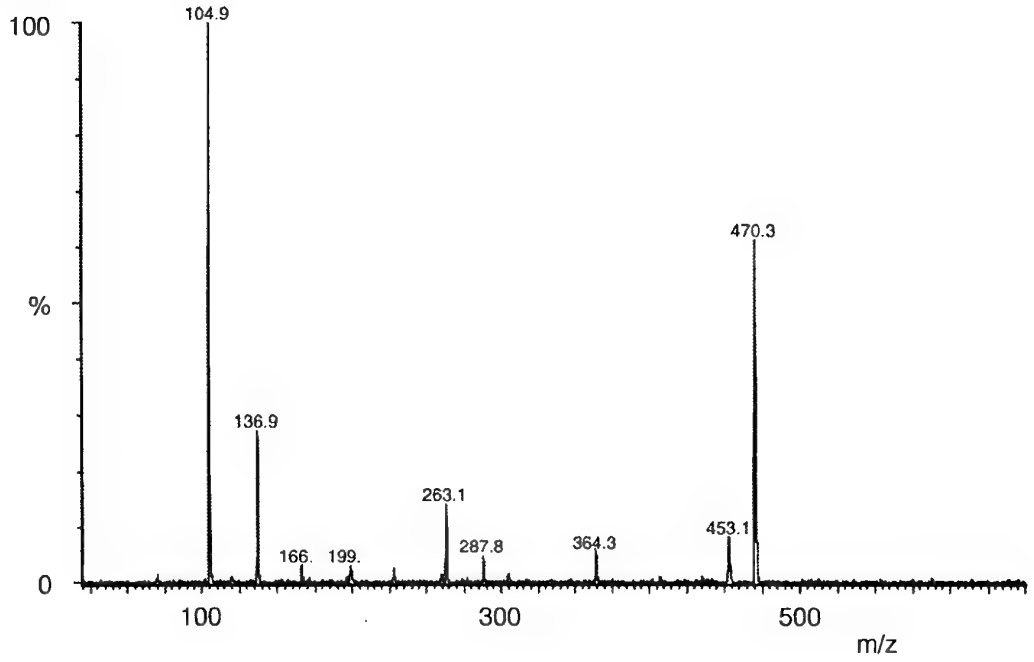


Figure 31. Tandem MS spectrum for molecular ion $MH^+$ (*m/z* 470) of synthetic (S-HETE)Cys-Pro-Phe; *m/z* 453 ($MH^+ - NH_3$), 137.1 ($HOCH_2CH_2SCH_2CH_2S^+$), 104.9 ($HOCH_2CH_2SCH_2CH_2^+$).

In view of the observed low minimum detectable concentration (*vide supra*), we applied this procedure to analysis of the blood samples taken from the nine Iranian victims from the Iran-Iraq conflict (see also Subsection IV.2.7). In all cases, except for the blank sample, the tripeptide could be detected (see Figure 32 for an example). Estimated exposure levels ranged from 0.4 to 1.8 μM which corresponds with the estimated levels determined with the modified Edman degradation (0.3-2 μM; see Subsection IV.2.7).

Next, experiments were carried out for optimization of the enzymatic cleavage of albumin in order to generate the tripeptide. We found that it was important to use relatively large amounts of pronase, *i.e.* pronase/albumin 1/3 (w/w). When lowering the ratio between pronase and albumin, the formation of the tetrapeptide Gln-(S-HETE)Cys-Pro-Phe was increased, which has less favourable properties for LC-tandem MS analysis. We also found, to our surprise, that less tripeptide was formed when the amount of digested albumin was increased from 3 to 10 mg, in the same volume and with the same enzyme/albumine ratio of 1/3. Probably, the protein concentration was too high in the sample, thereby preventing an optimal cleavage of albumin. When 1 mg instead of 3 mg of albumin was digested, the amount of tripeptide was 3 times less. It was also found that incubation for 2.5 h gave the highest yields of tripeptide. After 0.5 h, a considerable amount of tripeptide had already been formed which was only slightly less than after incubation for 2.5 h. After 3.5 h, the concentration of the tripeptide had decreased significantly and the dipeptide was present in larger amounts.

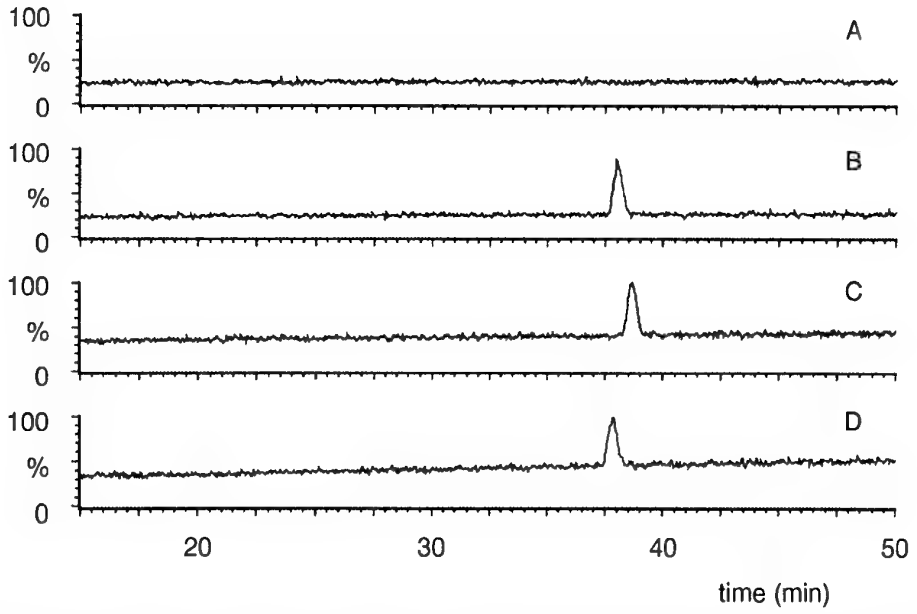


Figure 32. LC-tandem MS analysis of (S-HETE)Cys-Pro-Phe in a pronase digest of albumin, using the multiple reaction monitoring scanning mode for the transition *m/z* 470 ($MH^+$) → 105. Albumin was isolated from non-exposed human blood (A), from human blood that was exposed to 1 μM of sulfur mustard (B), and from blood taken from two Iranian victims (#9 and #8, see Table 2 in Subsection IV.2.7) 8-9 days after exposure to sulfur mustard (C and D, respectively).

Finally, experiments were carried out for optimization of the analysis of the tripeptide in pronase digests. After digestion of an albumin sample isolated from blood which had been exposed to [$^{14}$C]sulfur mustard (1 mM), it was established that elution of the pronase digest on a Sep-pak C18 cartridge led to a considerable clean-up of the sample. Elution was performed with 0.1% TFA/$H_2O$, 0.1% TFA/10% $CH_3CN$, 0.1% TFA/20% $CH_3CN$, and finally 0.1% TFA/40% $CH_3CN$. The fast eluting compounds (amino acids, dipeptides) were present in the 0.1% TFA/$H_2O$ and the 0.1% TFA/10% $CH_3CN$ fractions. The tripeptide was present in the 40% $CH_3CN$ layer (see Figure 33). The synthetic alkylated tripeptide coeluted with the tripeptide in the digest. It was established that the recovery of the tripeptide after filtration was 100%; the recovery of the Sep-pak procedure was 87%.

Use of filters with a cut-off of 3 kDa instead of filters with cut-off of 10 kDa for removal of the enzyme after digestion did not improve the analysis of the tripeptide.

When a Lichrosorb C18 (5 μm) microcolumn was used, instead of a PRP microcolumn, as an HPLC column, and by slight modification of the gradient, the tripeptide could be detected more sensitively. The detection limit for LC-tandem MS analysis of the standard was in this case 4 pg. With this improved procedure, including the Sep-pak C18 clean-up step, *we succeeded to detect an exposure level of 10 nM, using 3 mg of albumin, which is present in 120 μl of human blood (see Figure 34).* It was estimated from the peak areas for the tripeptides in the LC-tandem MS spectra that the relationship between exposure level (10 μM – 10 nM) and adduct level is still linear down to this concentration (see also Subsection IV.5.2). The experiment was repeated, using an albumin sample from a more recent series of exposures. Essentially, the

same results were obtained. In future experiments, it will be attempted to analyze the tripeptide even more sensitively (in order to lower the minimum detectable concentration) by using Fourier transform ion cyclotron resonance MS (FT-ICR-MS). These experiments will be carried out in the Environmental Molecular Sciences Laboratory (EMSL; Richland, Washington) and in the laboratory of Thermoquest (Bremen, Germany).

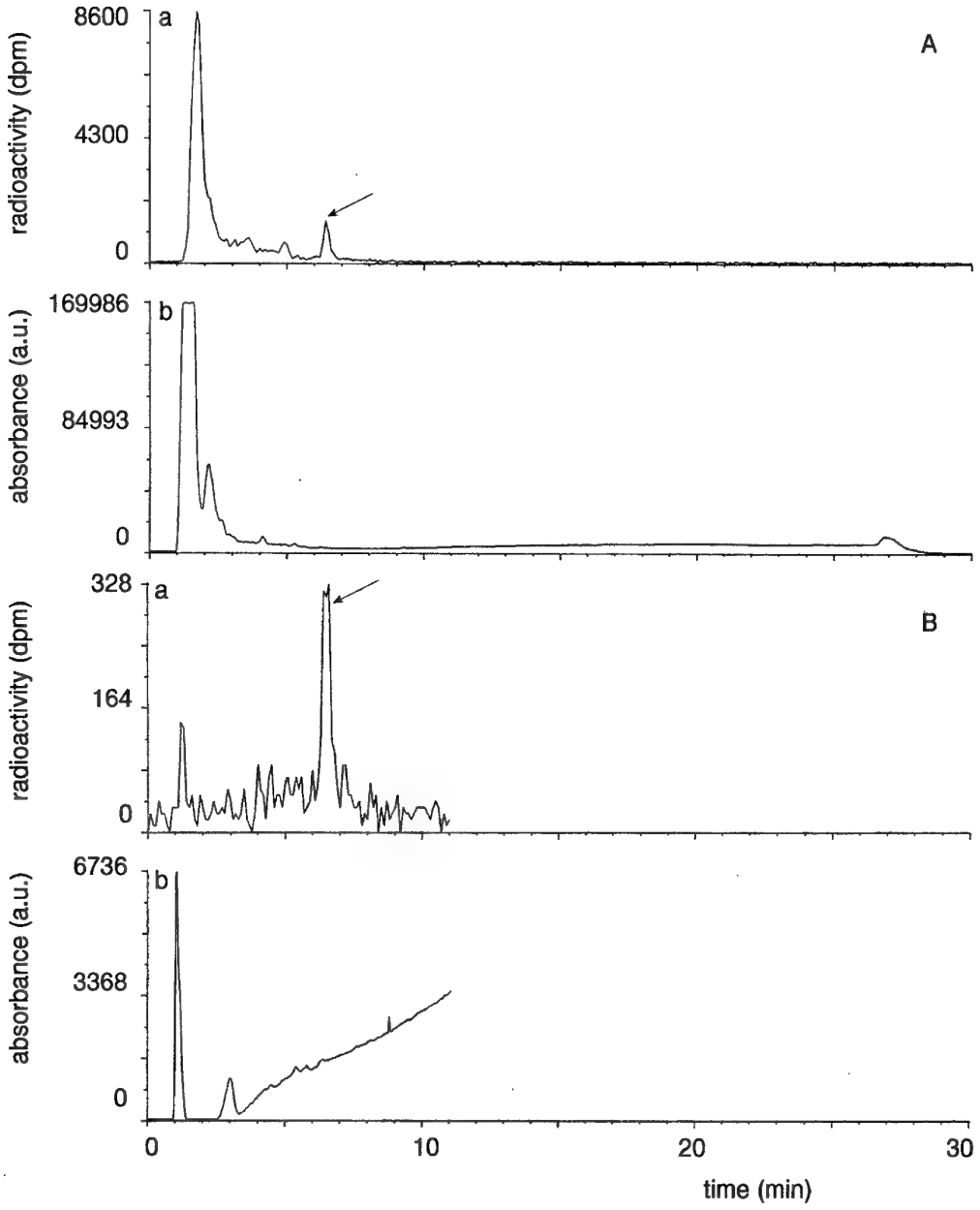


Figure 33. HPLC analysis (PepRPC 5/5 column) of (S-HETE)Cys-Pro-Phe (arrow) in a pronase digest of albumin isolated from human blood that was exposed to 1 mM $^{14}$C-sulfur mustard before (A) and after purification on a Sep-pak C18 cartridge (B). a, detection of radioactivity; b, UV detection (214 nm). Eluent (flow 1 ml/min): 0.1% trifluoroacetic acid with a linear gradient to acetonitrile/water/trifluoroacetic acid 80/20/0.1 (v/v/v) in 20 min.

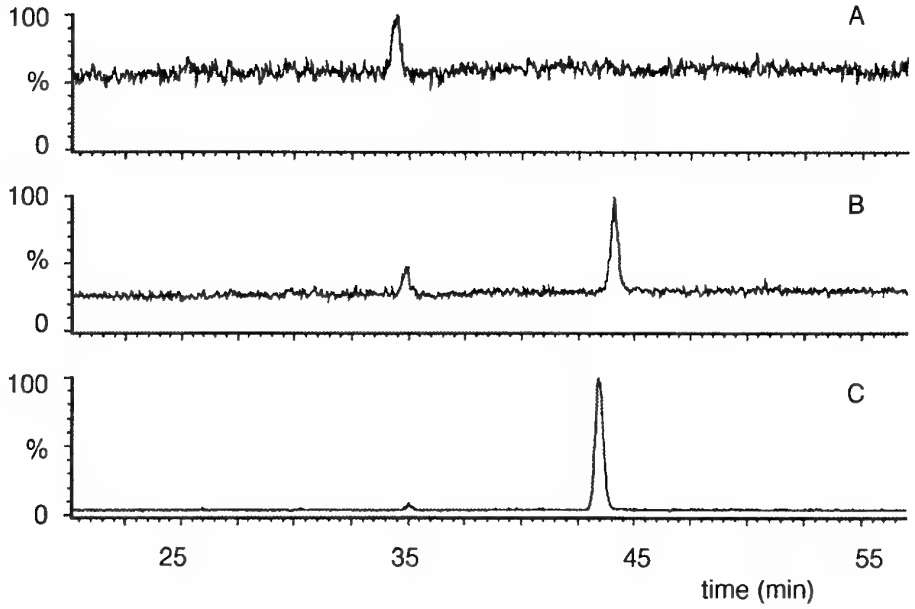


Figure 34. LC-tandem MS analysis of (S-HETE)Cys-Pro-Phe in a pronase digest of albumin (3 mg) after purification on Sep-pak, using the multiple reaction monitoring scanning mode for the transition *m/z* 470 ($MH^+$) → 105. Albumin was isolated from non-exposed human blood (A) and from human blood that was exposed to 10 nM (B) or 100 nM (C) of sulfur mustard.

### IV.5.5 Antibodies against the albumin T5 fragment containing a cysteine-sulfur mustard adduct

We immunized mice with the synthesized peptide hapten corresponding to the fragment T5 of human albumin containing an alkylated cysteine, described in Subsection IV.5.3. Subsequently, these mice were used for fusion experiments with the following results. Clones were selected on their capacity to produce antibodies with specificity for human albumin treated with 100 µM sulfur mustard, trypsinized human albumin treated with 1 mM sulfur mustard or to the peptide hapten used for immunization. Originally, several clones could be selected which showed some specificity for the alkylated albumin, the trypsinized alkylated albumin and/or peptide hapten. Unfortunately, in the further selection process all these clones lost their specificity for alkylated human albumin. Only in a few cases (six) stable clones could be obtained with some specificity for the alkylated peptide hapten in comparison to the non-alkylated peptide. The specificity of the antibodies against the alkylated peptide was not high enough to demonstrate the presence of the alkylated peptide after trypsinisation of sulfur mustard exposed albumin.

### IV.5.6 Feasibility of the modified Edman degradation on alkylated albumin for selective cleavage of the alkylated N-terminal aspartic acid

It was investigated whether the amino function of the N-terminal aspartic acid residue in albumin is alkylated by sulfur mustard. To this purpose the adduct was chemically synthesized by reaction of di-O-tert-butyl aspartic acid with the readily accessible 2-(2-tert-

butyloxyethylthio)ethyl chloride (Noort *et al.*, 1995). The fully protected adduct was obtained after purification by silica gel column chromatography. Subsequent treatment with TFA/$H_20$ afforded N-HETE-aspartic acid in reasonable yield (64%); $^1$H NMR and mass spectrometric data were in accordance with the proposed structure. Derivatization with pentafluorophenyl isothiocyanate led to the formation of a compound with the expected molecular mass (*m/z* 444), according to MS analysis with application of the sample directly on the MS probe. However, this compound could not be analyzed after GC-MS analysis, even not after derivatization with MTBSTFA. Probably, the free carboxyl group of the aspartic acid moiety is not properly derivatized by MTBSTFA. Otherwise, the silylated derivative is not stable enough to allow GC-MS analysis. Further attempts, *i.e.*, modified Edman degradation of albumin from human blood which had been exposed to sulfur mustard, were not undertaken.

## IV.6 Immunochemical and mass spectrometric detection of keratin adducts: exploratory research

### IV.6.1 Introduction

Firstly, steps similar to those for albumin adducts (see Section IV.5) were taken in order to develop an immunochemical assay for the detection of sulfur mustard adducts with keratin, *i.e.*, the most abundant protein present in human epidermis and stratum corneum. Binding of the agent to the protein was quantitated by using [$^{14}$C]sulfur mustard and keratin that had been exposed to sulfur mustard was hydrolyzed enzymatically. Although the latter approach did not give satisfactory results, results obtained in the first series of experiments provided sufficient information both to design and synthesize promising haptens for raising antibodies against keratin exposed to sulfur mustard and to develop a mass spectrometric analysis of sulfur mustard adducts formed with the protein. Experiments aiming at optimization of the isolation of sulfur mustard adducts from keratin and subsequent MS analysis have been performed. The procedure was applied to keratin extracts of human skin exposed to sulfur mustard vapor. Several monoclonal antibodies obtained from immunization with the synthesized haptens showed some specificity on the horny layer of human skin exposed to sulfur mustard. These results open the way for direct immunochemical detection of skin sites contaminated by sulfur mustard.

### IV.6.2 Isolation, purification and enzymatic hydrolysis of keratin from human callus that was exposed to sulfur mustard

First, keratin was isolated from human callus by salt extraction and was subsequently purified by means of gel filtration according to procedures reported in literature (Sun and Green, 1978). The amino acid composition of the isolated keratin was in reasonable agreement with literature data (Fuchs and Green, 1978; see Table 14).

Subsequently, a suspension of human callus (0.5 g/ml) in 0.9% NaCl/isopropanol (1/1, v/v) was exposed to various concentrations of [$^{14}$C]sulfur mustard for 6 h at 37 °C. The extracted keratin fractions contained ca. 15-20% of the added radioactivity, in each case (see Table 15). Upon purification on a G-75 column ca. 25% of the activity bound to keratin was eluted with a Tris buffer (10 mM Tris.HCl, 10 mM dithiothreitol, 0.5% SDS, pH 7.6) as low molecular material, probably thiodiglycol.

Table 14. Amino acid composition (mole%) of isolated keratin

| Amino acid | isolated keratin | literature data[a] |
|---|---|---|
| Gly | 23.4 | 20.9 |
| Ser | 13.4 | 12.4 |
| Glx | 9.5 | 13.3 |
| Asx | 8.9 | 8.5 |
| Leu | 7.8 | 8.4 |
| Thr | 6.1 | 3.7 |
| Lys | 5.9 | 4.5 |
| Arg | 5.1 | 5.5 |
| Ile | 4.5 | 4.0 |
| Ala | 4.0 | 5.5 |
| Phe | 3.6 | 3.2 |
| Val | 3.2 | 3.7 |
| Pro | 1.4 | 1.3 |
| Met | 1.2 | 1.5 |
| His | 0.9 | 1.5 |
| Tyr | 0.9 | 3.6 |
| Cys | not determined | not determined |

[a] Fuchs and Green, 1978.

Several proteases, *i.e.*, trypsin, α-chymotrypsin and V8 protease, were used in order to identify alkylated sites in keratin after exposure of human callus to sulfur mustard. Keratin isolated from human callus that had been exposed to $^{14}$C-sulfur mustard was suspended (3.5 mg/ml) in Cleveland's buffer (125 mM Tris.HCl, 0.5% SDS, 10% glycerol, pH 6.8), a borate buffer (50 mM KCl, 50 mM disodium tetraborate, 1 mM dithiothreitol, pH 9.2, diluted 50 times with water) or a citrate buffer (10 mM sodium citrate, acidified with aqueous HCl to pH 2.6) and incubated with trypsin, immobilized trypsin, α-chymotrypsin or V8-protease at 37 °C for 1 h. The filtrates obtained after passing the incubation mixtures over a UF-2 filter (cut-off 10 kDa) were analyzed by means of HPLC with radiometric detection. No radioactive peptide material could be detected, indicating that amino acids or peptides containing a sulfur mustard adduct had not been released.

Table 15. Binding of [$^{14}$C]sulfur mustard to keratin upon treatment of human callus suspended in 0.9% NaCl (1 g/ml) with various concentrations of the agent in an equal volume of isopropanol

| Concentration [$^{14}$C]sulfur mustard (μM) | [$^{14}$C]Sulfur mustard bound to keratin (% of total radioactivity added) |
|---|---|
| 0.1 | 17 |
| 1.0 | 15 |
| 10 | 15 |
| 100 | 20 |
| 1,000 | 20 |
| 10,000 | 22 |

### IV.6.3 Isolation and derivatization of thiodiglycol after alkaline hydrolysis of keratin

Release of radioactivity upon purification at pH 7.6 of keratin that was exposed to [$^{14}$C]sulfur mustard (see previous subsection) suggests that part of the adducts formed with keratin are readily split off from the protein. It is known that keratin contains a large number of glutamic and aspartic acid residues (cf. Table 14). Consequently, it can be expected that upon exposure to sulfur mustard these residues are converted into esters of thiodiglycol which are readily hydrolyzed with mild base. To check this hypothesis a purified keratin sample, isolated from human callus exposed to $^{14}$C-sulfur mustard, was incubated with aqueous NaOH (0.5 M). After chromatography of the mixture on a G75 column, only 20% of total radioactivity coincided with keratin, whereas 80% of total radioactivity eluted as material with low molecular mass. One of the fractions containing the low molecular material was further worked-up with a combined ChemElut/Sep-Pak C18 extraction. TLC analysis with radiometric detection of the obtained extract (in ethyl acetate) showed that the radioactive component coeluted with thiodiglycol. In a later stage, the reaction mixture was filtered over a UF-2 filter (cut-off 10 kDa) for isolation of thiodiglycol, which is a less laborious procedure. It was established that only small losses (< 10%) of [$^{14}$C]thiodiglycol occurred during filtration and subsequent evaporation of the filtrate to dryness.

These results open the way for sensitive mass spectrometric detection of sulfur mustard exposure of skin, since a method for derivatization of thiodiglycol and subsequent sensitive analysis of the derivative has been reported in literature (Black and Read, 1988). In preliminary experiments, thiodiglycol obtained from keratin that was exposed to [$^{14}$C]sulfur mustard was derivatized with pentafluorobenzoyl chloride according to this procedure. HPLC analysis with radiometric detection revealed one single radioactive compound, which coeluted with a synthetic standard of the bis(pentafluorobenzoyl) ester of thiodiglycol.

Thiodiglycol obtained by alkaline treatment of keratin (5 mg), isolated from human callus which had been exposed to various concentrations (10 – 1000 µM) of sulfur mustard, could be analyzed by GC-MS, after derivatization with pentafluorobenzoyl chloride (see Figure 35). Keratin isolated from human callus which had been exposed to sulfur mustard-$d_8$ (10 mM) was used as an internal standard. The detection limit for bis (pentafluorobenzoyl)thiodiglycol was determined to be 5 pg, whereas this derivative could still be analyzed after exposure of human callus to 10 µM of sulfur mustard. In a blank keratin sample the derivative was not detected.

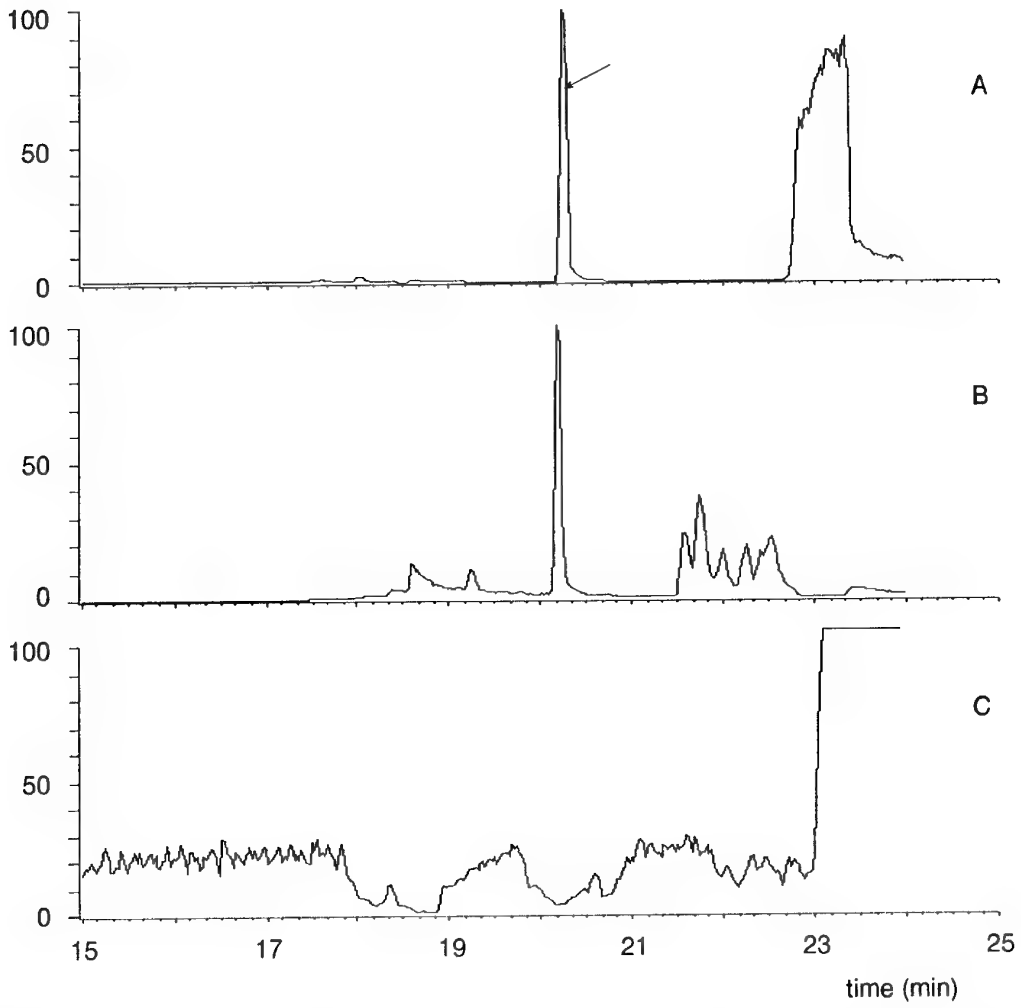


Figure 35. GC-NCI/MS analysis of the bis(pentafluorobenzoyl) ester of thiodiglycol obtained by alkaline treatment of keratin isolated from human callus that had been exposed to 0.5 mM sulfur mustard (a), to 10 mM sulfur mustard-$d_8$ (b), and of keratin isolated from non-exposed human callus (c). Ion chromatograms were recorded after monitoring for *m/z* 510 ($M^-$, analyte) and 518 ($M^-$, internal standard).

In order to enable direct detection of sulfur mustard adducts in the skin, the hydrolysis of the thiodiglycol esters should be performed at mild conditions. Several approaches were followed in order to achieve substantial release of thiodiglycol at such conditions. First, the effect of the pH was determined on the release in aqueous NaOH of thiodiglycol from keratin isolated from callus that had been exposed to [$^{14}$C]sulfur mustard. After incubation at pH 7, 9, 10 or 11 for 1 h at room temperature, only 10% of the total radioactivity was found in the filtrate obtained after filtration of the incubation mixture over a UF 10 kDa filter. Incubation at high pH, *i.e.*, 12 or 13, is necessary to induce a substantial release of thiodiglycol, *i.e.*, 37% and 80%, respectively. Neither addition of SDS (0.5%), urea (1 M), histidine (10 mM) or sodium phosphate (10 mM) to aqueous NaOH (pH 9) nor treatment (1 h, room temperature) with aqueous $NH_4OH$ at pH 9 did result in a significant increase in released radioactivity.

In addition, aminolysis by the primary amines isopropylamine, ethanolamine, octylamine, benzylamine, decylamine and dodecylamine (10 mM in water) was investigated for release of thiodiglycol instead of aqueous NaOH. Unfortunately, incubation with neither of these amines increased release of radioactivity.

Finally, it was attempted to release thiodiglycol from adducted keratin by catalyzing the ester hydrolysis with porcine liver esterase. After treatment at room temperature for 3 h, comparable amounts of total radioactivity (10%) were detected in the filtrate of the sample containing the esterase and in the filtrate of the blank sample. The experiment was repeated at 37 °C. The amount of radioactivity which was detected in the filtrate was still 10% after 4 h of incubation, but amounted to 50% after 36 h of incubation. However, the latter conditions are not suitable for practical application.

In conclusion, release of thiodiglycol from adducted keratin should be performed at high pH, *i.e.*, 0.5 N NaOH, when following this approach for a mass spectrometric detection of sulfur mustard exposure of skin.

The same procedure could be applied to keratin extracts of human skin that was exposed to saturated sulfur mustard vapor *in vitro*. Small pieces of human skin were exposed to saturated sulfur mustard vapor (0, 2, 4 or 8 min) according to the method described in Subsection III.3.6. Keratin extracts were obtained according to Mol (2000). After treatment with 0.5 M NaOH, thiodiglycol was isolated and analyzed according to the method described above. A linear relationship was observed between the amount of liberated thiodiglycol and exposure time (see Figure 36).

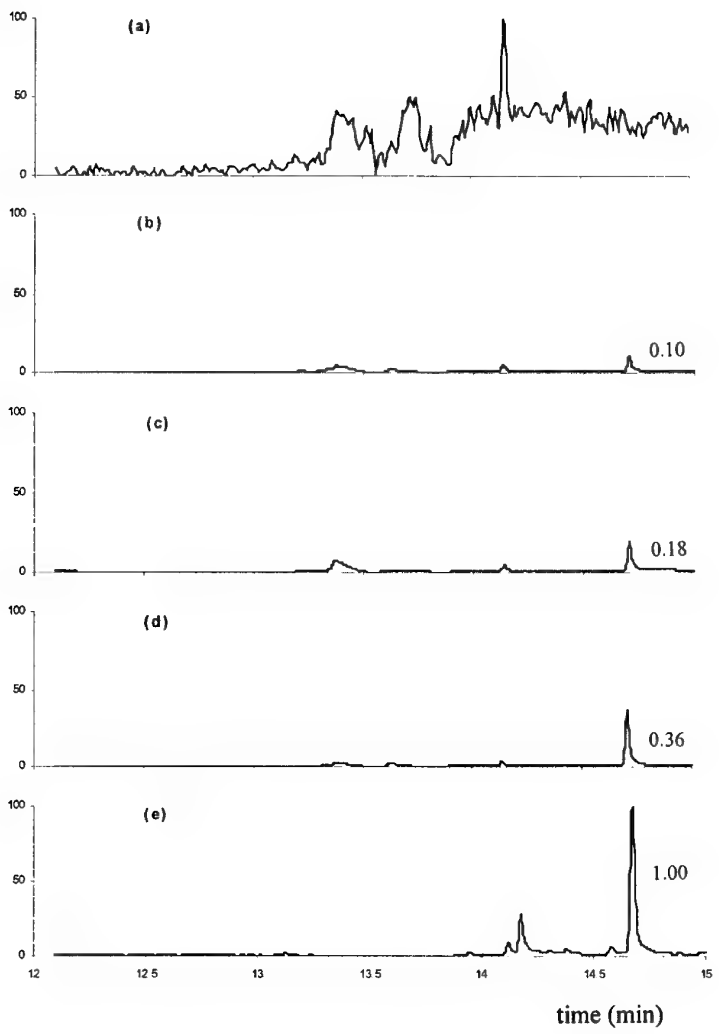


Figure 36. GC-NCI/MS analysis of the bis(pentafluorobenzoyl) ester of thiodiglycol obtained by alkaline treatment of keratin extracts isolated from human skin that had been exposed to saturated sulfur mustard vapor at 20 °C. Exposure times were varied: (a) 0 min; (b) 2 min; (c) 4 min; (d) 8 min. Ion chromatograms a-d were recorded after monitoring for *m/z* 510 ($M^-$, analyte) and ion chromatogram e was recorded after monitoring for *m/z* 518 ($M^-$, internal standard). Peak areas of the analytes (b-d) relative to that of the internal standard (e) are given.

### IV.6.4 Synthesis of haptens containing a glutamine- or asparagine-sulfur mustard adduct

Since we found that glutamic acid and aspartic acid residues in keratin are efficiently alkylated by sulfur mustard (see previous subsection), we intended to synthesize partial sequences of keratin, containing a sulfur mustard adduct of these amino acids, which can be used as haptens for raising antibodies. It can be expected, however, that the thiodiglycol esters are not stable during immunization. Therefore, we decided to employ the corresponding amides (see Figure 37 for chemical structures of a thiodiglycol ester and a corresponding amide). As a first approach, we tried to synthesize these compounds by solid-phase synthesis of a peptide

containing a glutamic or aspartic acid residue protected with an allyl function, which can selectively be removed by reduction on a palladium catalyst. Subsequent coupling with 2-(2-aminoethylthio)ethanol would yield the desired compound. However, we did not succeed in removing the allyl function from the peptide when it was still attached to the solid support, despite several efforts, using palladium catalysts from various manufacturers.

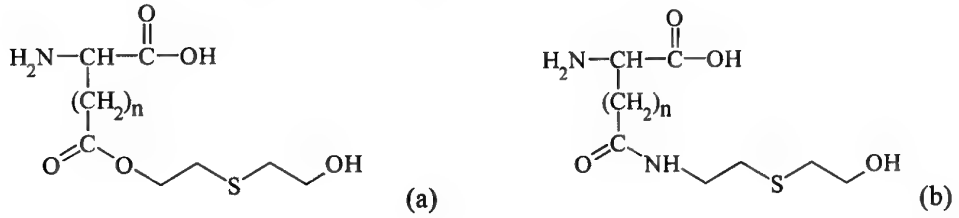


Figure 37. Chemical structures of the thiodiglycol esters (a) and amides (b) of aspartic acid (n=1) and glutamic acid (n=2).

We then decided to synthesize a building block which could be incorporated into the peptide during solid-phase synthesis. As starting material we chose the commercially available Boc-Glu-OtBu and Boc-Asp-OtBu, containing a free side-chain carboxylic acid group. Coupling with 2-(2-aminoethylthio)ethanol under the agency of PyBOP and NMM afforded, after purification on silica gel, the glutamine/asparagine amide derivatives in moderate yield. Deprotection with TFA, followed by introduction of the Fmoc group and purification by means of gel filtration on Sephadex LH-20 gave the desired building blocks for solid-phase synthesis. The following peptides derived from partial sequences of end domains of human keratins K5 and K14, which are probably more accessible than other part of the proteins, were synthesized with these building blocks:

1. G-V-V-S-T-H-(Nω-HETE)Q-Q-V-L-R-T-K-N-K, derived from human keratin K14
2. G-I-Q-(Nω-HETE)Q-V-T-V-N-Q-S-L-L-T-P-L-N-K, derived from human keratin K5
3. G-V-M-(Nω-HETE)N-V-H-D-G-K-V-V-S-T-H-E-K, derived from human keratin K14

Electrospray MS analysis showed the correct mass in each case. The peptides were used for the raising of antibodies. The three native sequences were also synthesized and served as reference compounds in immunochemical experiments with antibodies raised against the three peptides containing Nω-HETE-glutamine or Nω-HETE-asparagine.

### IV.6.5 Antibodies against partial sequences of containing a glutamine- or asparagine-sulfur mustard adduct

Mice were immunized with 3 partial sequences of keratin containing a glutamine- or asparagine-sulfur mustard adduct as described in the previous subsection, in three different series. A mixture of peptide 1 and 2 was used in series 1, only peptide 3 in series 2 and a mixture of all 3 peptides in series 3. The spleen of one mouse of each series was selected for three separate fusion experiments carried out simultaneously. Clones were selected in a direct ELISA on their specificity for keratin isolated from human callus exposed to 50 and 100 μM sulfur mustard. The results of the four clones giving the best response in the direct ELISA are presented in Table 16.

Table 16. Antibody specificities of clones obtained from a fusion after immunization with one hapten or a mixture of two or three partial sequences of keratin containing glutamine (1,2)- or asparagine (3)-sulfur mustard adduct. Supernatants of cultures in a 1:5 dilution were assayed in a direct ELISA on keratin from human callus treated with 0, 50 or 100 µM sulfur mustard. Fluorescence (in arbitrary units) is presented as a measure for antibody binding.

| Clone | Peptides used for immunization | Antibody response against keratin exposed to sulfur mustard solution of | | |
|---|---|---|---|---|
| | | 0 µM | 50 µM | 100 µM |
| 3.2G8 | 1+2+3 | 300 | 1400 | 2400 |
| 1.2B6 | 1+2 | 1400 | 3900 | 4300 |
| 1.3C2 | 1+2 | 300 | 1500 | 2700 |
| 2.3D9 | 3 | 300 | 2300 | 3400 |

Several of these clones have been stored frozen. After a certain period of time, the clones were cultured again and analyzed for antibody-specificity. Nine clones were selected for further characterization and subcloning. So far, 32 monoclonal clones, all originating from clone 1.3C2, have been selected of which antibodies showed specificity against keratin treated with 50 µM sulfur mustard. These 32 monoclonal antibodies were tested in an immunofluorescence experiment with human skin exposed to 0, 50 and 100 µM sulfur mustard (30 min at 27 °C) or to saturated sulfur mustard vapor (1 min at 27 °C; Ct value of 1040 $mg.min.m^{-3}$). Cross-sections were prepared of the sulfur-mustard exposed skin samples and processed with the antibodies. The binding of the antibodies to the horny layer was detected by binding of a second antibody conjugated with the fluorescing group FITC, which was directed against the first antibody. The DNA in the epidermal and dermal cells was counterstained with propidium iodide for localization of the DNA containing cells. The results are summarized in Table 17.

A large number of the monoclonal antibodies (18 out of 32) showed some specificity to sulfur mustard adducts in the horny layer of sulfur mustard exposed skin as indicated by the higher fluorescence intensity in comparison with the background intensity found in non-exposed skin (0 µM sulfur mustard). In general, the vapor-exposed skin samples showed the highest response. Monoclonal antibody 1H10 was tested at higher dilutions in an attempt to lower the a-specific binding of antibodies to non-exposed skin and to increase the sensitivity of this immunofluorescence procedure for detection of exposure of human skin to sulfur mustard in the horny layer and possibly in other keratin-containing parts of the epidermal layer. As a preliminary result, exposure of human skin to a solution of 100 µM sulfur mustard or to saturated sulfur mustard vapor during 1 min was analyzed by using a 50-fold diluted supernatant. Photographs of skin cross-sections are given in Figure 38. Sulfur mustard adducts are clearly detected in the horny layer wherease DNA staining is found in the epidermis. Hardly any fluorescence due to antibody treatment is measured over the non-exposed skin cross-section at the conditions of this preliminary experiment. *It should be emphasized that the antibodies were directly applied to the human skin cross-sections without pre-conditioning of the samples. This opens the way for development of a detection kit that can be applied directly to skin of human beings who are supposedly contaminated by sulfur mustard.*

Table 17. Immunofluorescence microscopy on cross-sections of human skin exposed to 0, 50 and 100 µM sulfur mustard (30 min at 27 °C) or to saturated sulfur mustard vapor (1 min at 27 °C; Ct: 1040 mg.min.m$^{-3}$), showing binding of 32 monoclonal antibodies[a] to the horny layer. The antibodies raised against partial sequences of keratin containing a glutamine- or asparagine-sulfur mustard adduct were selected for specificity to sulfur mustard-keratin adducts in a direct ELISA.

| Monoclonal antibody | Fluorescence intensity[b] for exposure to Sulfur mustard solution of 0 µM | 50 µM | 100 µM | Sulfur mustard vapor |
|---|---|---|---|---|
| 1B12 | + | ++ | + - +++[c] | |
| 1A10 | ++ | + | + | ++ |
| 3H12 | + | | + | ++ |
| 2D11 | + | + | + | ++ |
| 1C12 | + | + | + | + |
| 1C11 | + | ++ | ++ | +++ |
| 2G10 | + | | + | ++ |
| 2G8 | + | + | + | ++ |
| 1H11 | + | | + | + |
| 1H8 | + | | + | + |
| 1D8 | + | | + | + |
| 1C5 | + | | + | |
| 1B2 | + | | + | + |
| 1B3 | + | | + | + |
| 1B4 | + | | + | + |
| 1A8 | + | | + | |
| 1G2 | ++ | ++ | + | +++ |
| 2C3 | + | ++ | +++ | +++ |
| 1H10 | + | ++ | +++ | +++ |
| 1E11 | ± | + | + | ++ |
| 1E4 | ++ | ++± | +++ | +++ |
| 1B6 | ++ | +++ | +++ | +++ |
| 2F6 | ++ | +++ | +++ | |
| 1F2 | +± | ++ | +++ | +++ |
| 2F8 | +++ | + | | ++ - +++[c] |
| 2D3 | +± | | ++ | ++ |
| 2G7 | + | + | + | ++ |
| 2B1 | ± | ± | ± | ± |
| 1D9 | + | ++ | + | ++ |
| 1C7 | + | | + | ++ |
| 1C3 | + | ++ | ++ | + - ++[c] |
| 1C2 | ± | ± | ± - +[c] | + |

[a] All supernatants of the monoclonals have been applied in a 1:1 dilution. Binding of the antibodies to the horny layer was detected by binding of a second antibody conjugated with the fluorescing group FITC which was directed against the first antibody.

[b] The fluorescence intensity above the horny layer estimated by eye is indicated, in increasing order, by ±, +, +±, ++, and so on.

[c] The fluorescence intensity varied within one preparation.

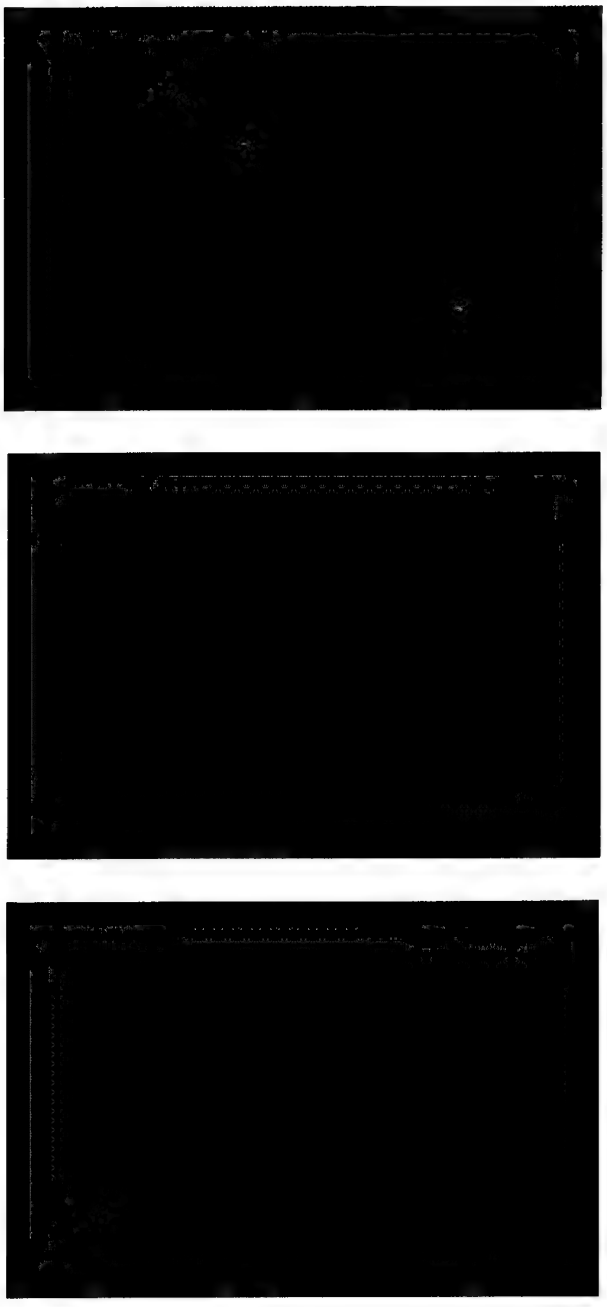


Figure 38. Immunofluorescence microscopy of a cross-section of human skin exposed to saturated sulfur mustard vapor (1 min at 27 °C; Ct 1040 mg.min.m$^{-3}$; A) or to a solution of sulfur mustard (100 $\mu$M, 30 min at 27 °C; B) and of non-exposed skin (C), using monoclonal antibody 1H10, directed against sulfur mustard adducts to human keratin, as a 50-fold diluted supernatant. The photographs are composed from an image obtained for FITC fluorescence (mainly emanating from the horny layer; green) and from an image obtained for propidium iodide fluorescence representing DNA (red) in the same cross-section.

## IV.7 Cross-reactivity of antibodies for sulfur mustard adducts with hemoglobin, albumin and keratin

As described in Subsection IV.4.4, antibodies raised against peptide haptens containing a histidine-sulfur mustard adduct showed specificity for both hemoglobin treated with 50 µM sulfur mustard and keratin treated with 50 or 500 µM sulfur mustard. In case of clone 190-2H12, the specificity for alkylated keratin seemed to be even higher than for alkylated hemoglobin. This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

Since we were not very successful in raising antibodies against alkylated human albumin so far, we studied whether antibodies which recognize adducts to keratin and/or hemoglobin cross-react with adducts to albumin. One of the clones raised against alkylated keratin (1H10) with a positive response on sulfur mustard-exposed skin was brought in culture and checked for cross-reactivity with albumin exposed to 100 µM sulfur mustard. The antibodies of this clone produced a 20-fold higher fluorescence in a direct ELISA with keratin exposed to 50 µM sulfur mustard than with unexposed keratin. However, these antibodies did not show any cross-reactivity with albumin exposed to 100 µM sulfur mustard. Only a slightly increased fluorescence (1.5-fold) was observed with trypsinized alkylated albumin in comparison to trypsinized non-alkylated albumin. In addition, these antibodies did not recognize the hapten used for raising antibodies against the alkylated T5-fragment of albumin.

Furthermore, we tested 33 clones (obtained after subcloning of clones 183-5B7, 183-3D5 and 190-4A3 presented in Table 12 in Subsection IV.4.4), selected from fusions from mice immunized with peptide haptens containing a histidine-sulfur mustard adduct and with amino acid sequences present in human hemoglobin, on cross-reactivity to adducts on albumin. Undiluted supernatants of cultures of four of these clones showed some specificity for alkylated albumin. However, none of them was positive after repeated testing with diluted supernatant on alkylated albumin. Only one of these was still slightly positive on trypsinized alkylated albumin.

# V DISCUSSION

## Synthesis of radioactively labeled sulfur mustard

Radioactively labeled sulfur mustard has advantageously been used in various series of experiments described in this report. In previous studies we synthesized the $^{35}S$-labeled agent for similar purposes. However, the synthesis of [$^{35}S$]sulfur mustard was often accompanied with difficulties which were probably caused by the presence of impurities in the synthesized hydrogen [$^{35}S$]sulfide. A more reliable synthetic route could be developed for radioactively labeled sulfur mustard containing a $^{14}C$-label instead of a $^{35}S$-label. An additional advantage of this new product is the much longer half life of its radioisotope. The crucial step in the new synthetic route is the reaction of [$^{14}C$]bromoethanol with 2-mercaptoethanol. The major by-product formed in this reaction is the disulfide analogue of thiodiglycol. Since a radioactive precursor is not involved in the formation of this by-product, it has no influence on the radiochemical purity of the end product. The method was further optimized by replacing the reagent for conversion of [$^{14}C$]thiodiglycol into [$^{14}C$]sulfur mustard, *i.e.*, thionylchloride, by concentrated hydrochloric acid. This resulted in a higher overall yield (56%), whereas further purification was not necessary.

## Development of two Standard Operating Procedures for determination of sulfur mustard adducts

Within the framework of a previous grant (Benschop and Van der Schans, 1995) two methods for diagnosis and dosimetry of exposure to sulfur mustard were sufficiently worked out to justify the development of SOPs to be applied in a well-equipped field hospital, *i.e.*, an immunoslotblot assay of sulfur mustard adducts to DNA in human blood and skin, and a GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin of human blood by using the modified Edman procedure. Moreover, the immunoslotblot assay was optimized in a twofold way, *i.e.*, for optimal simplicity and for optimal sensitivity. Development of these SOPs is one of the two major topics of the present grant and was performed in three phases: (i) simplification and optimization of the two methods, (ii) validation in animal experiments of the methods performed according to the final procedures, and (iii) description of the SOPs and performance of the procedures in a U.S. Army laboratory (MRICD) in order to demonstrate their practical applicability.

*Development of an immunochemical assay of sulfur mustard adducts to DNA as a Standard Operating Procedure*

The present experiments showed that several steps could be simplified and minimized: collection of a blood sample and/or skin biopsy, isolation of DNA, measurement of the concentration and denaturation of DNA, followed by the immunoslotblot procedure, involving blotting and crosslinking of the DNA on a nitrocellulose filter, a blocking step, treatment with 1st and 2nd antibody, addition of substrate and, finally, measurement of the chemiluminescence as a measure for the amount of sulfur mustard adducts to the DNA. The amount of blood required could be reduced from 1 ml to only 300 µl. In addition, sufficient amounts of DNA could be isolated from a human skin biopsy of 10-20 $mm^2$.

Although the DNA isolation procedure is still a time-consuming step, substantial reduction in time and labor could be achieved. Essentially, the lysis of the white blood cells could be accomplished in 1 h instead of by overnight incubation. Moreover, the repeated phenol/chloroform/isoamylalcohol extractions could be replaced by a simple protein precipitation step, whereas RNAse treatment carried out before the protein precipitation makes

destruction of RNAse by a proteinase K treatment redundant. In this way, the labor time for DNA isolation could be reduced from one and a half day to about 3 h. However, one has to keep in mind that an overnight incubation is still necessary for dissolution of the DNA pellet. Dissolution of the DNA pellet can be carried out in 1 to 2 h, when the DNA isolation can be carried out on fresh blood. In that case, it is estimated that the whole DNA isolation procedure is reduced to about 4–5 h. In this set-up, 20 samples can be handled simultaneously.

The use of a luminometer for the direct measurement of the chemiluminescence instead of the use of the combination of a photographic film and a densitometer for measurement of the blackening appeared to yield a significant reduction in time and labor for performance of the immunoslotblot assay (a half working day). In this set-up of the immunoslotblot procedure, 39 samples can be assayed in duplicate on one nitrocellulose filter, in addition to the standard DNA samples.

In addition to these modifications, which resulted in simplification and shortening of the assay, several improvements could be achieved with respect to the sensitivity. The accurate measurement of the concentration of DNA appeared to be essential due to the strong dependence of the chemiluminescence signal upon the amount of DNA blotted on the filter. The UV crosslinking on the nitrocellulose filter resulted in an approximately 10-fold enhancement of the chemiluminescence signal and, consequently, in an improvement of the sensitivity of the assay. The use of a luminometer for measurement of chemiluminescence instead of exposure to a photographic film circumvents the non-linear blackening characteristics of the films. In this way, a linear relationship was obtained for the chemiluminescence as a function of the sulfur mustard concentration with which double-stranded calf thymus DNA was treated. As a result of these modifications, chemiluminescence observed for double-stranded calf thymus DNA treated with 2.5 nM sulfur mustard was enhanced relative to that for untreated DNA, whereas the minimum detectable concentration in previous experiments was at about 10 nM sulfur mustard. The minimum detectable concentration in the modified assay showed some variation which may partly be due to day-to-day variations in the state of the chemiluminescence blotting detection system. Nevertheless, it could be derived that the lower detection limit was in a range of 8-40 amol N7-HETE-Gua/blot using 1 µg DNA. This corresponds to an adduct level of 3-13 N7-HETE-Gua/$10^9$ nucleotides.

At the start of this study the minimum detectable concentration of the immunoslotblot assay for exposure of human blood was 70 nM sulfur mustard. This corresponds to an adduct level of 300 N7-HETE-Gua/$10^9$ nucleotides. Due to the improvements mentioned above, it can be calculated that the minimum detectable concentration for *in vitro* exposure of human blood should be 0.7-3 nM sulfur mustard. However, the adduct levels detected at sulfur mustard concentrations in the range of 100 nM were much lower than expected. Therefore, a minimum detectable concentration of exposure of human blood *in vitro* down to 50 nM sulfur mustard is feasible. The immunoslotblot assay showed a rather large day-to-day variation, corresponding to an up to 2-fold difference in adduct level, which appeared to be mainly introduced during DNA isolation and denaturation. This inaccuracy of the procedure may partly explain the discrepancy observed between the presently determined adduct levels and the adduct levels determined previously after *in vitro* exposure of human blood (Benschop and Van der Schans, 1995).

The SOP in its current state, including the DNA isolation procedure, takes about 1.5 working days and two overnight incubation steps, i.e., for dissolution of the DNA pellet and for adsorption of the 1st antibody. In a shortenend mode of the SOP for the assay of blood samples, modifications were introduced in order to further speed up the procedure, aiming at a procedure as simple as possible that is suitable for performance in a field laboratory, while accepting some decrease in sensitivity and accuracy. For this purpose, dissolution of the DNA pellet was carried out for only

30 min, followed by a short centrifugation step to remove undissolved material. Moreover, several other steps in the procedure were skipped or shortened, including incubation with the 1st antibody, which was reduced to 2 h. The shortened procedure resulted in a lower sensitivity. Nevertheless, human blood exposed *in vitro* to 1 μM sulfur mustard is still detectable. In this way, 12 blood samples can be processed simultaneously and data can be generated within 9 h.

*Development of a GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin as a Standard Operating Procedure*

The first step in the procedure is the isolation of globin from blood, which takes approximately 2-3 h. Unfortunately, a straightforward approach of shortening the procedure by omitting this isolation step and treating the hemolysate with the modified Edman reagent did not lead to a detectable product. However, the duration of the second step, *i.e.*, treatment with the modified Edman reagent, could be considerably reduced without loosing sensitivity by performing the reaction for 2 h at 60 °C instead of overnight at room temperature, followed by 2 h at 45 °C. In addition, the work-up of the reaction mixture was somewhat shortened. As a result, the complete procedure, *i.e.*, isolation of globin, reaction with the modified Edman reagent, work-up, derivatization, and GC-NCI/MS analysis, can now be performed within one working day.

*In vitro* exposure of human blood to ≥ 0.1 μM sulfur mustard could be determined by using the modified Edman procedure as developed in our earlier studies (Benschop and Van der Schans, 1995). Previously, this method was sufficiently sensitive for retrospective detection of exposure to sulfur mustard of two victims from the Iran-Iraq conflict (Benschop and Van der Schans, 1995; Benschop *et al.*, 1997). We now report the detection of the adduct of sulfur mustard to the N-terminal valine residue of globin in blood samples taken from nine more Iranian victims, who were exposed to sulfur mustard 8-9 days earlier. Adduct levels comparable with *in vitro* exposure of human blood to a sulfur mustard concentration ranging from 0.3-2 μM were found in two series of determinations. Unfortunately, the adduct levels found in these two series of experiments differ considerably, up to 7-fold although globin isolated from blood that had been exposed to sulfur mustard-$d_8$ was used as an internal standard in all analyses. This result is probably due to improper functioning (*i.e.*, for quantitative analysis) of the GC-MS apparatus used for the analyses, as turned out from analyses performed later on in this project. Nevertheless, these results could be confirmed by our newly developed analyses based on the detection of an alkylated tripeptide in a pronase digest of albumin isolated from these blood samples (*vide infra*), which showed adducts levels comparable with albumin adduct levels found after *in vitro* exposure of human blood to sulfur mustard concentrations ranging from 0.4-1.8 μM. We could not confirm these results from analysis of N7-HETE-Gua in white blood cells of the blood samples by using an immunochemical assay. This was due to coagulation or partial precipitation of the old blood samples (taken in 1986) which hampers proper isolation of DNA.

It was envisaged that some steps in the procedure could presumably be improved leading to a lowering of the minimum detectable concentration, *e.g.*, the processing of a larger sample of globin isolated from blood that was exposed to sulfur mustard, and GC-NCI/MS analysis of a larger fraction of the final sample obtained by the procedure. In a first series of experiments solid phase extraction procedures were used to purify the crude thiohydantoin obtained after treatment with the modified Edman reagent. This additional purification allowed to process a three-fold larger amount of globin without adverse effect on the GC-NCI/MS analysis. Processing of even larger amounts resulted in impurities in the final sample that hampered proper analysis. However, a further lowering of the minimum detectable concentration of the procedure for *in vitro* exposure of human blood was not achieved. In a second series of experiments, a TCT injection technique was used in the GC-NCI/MS analysis of the final

sample. Much larger injection volumes can be applied with this technique instead of a usual on-column injection (50-100 µl vs 1-3 µl), leading to a 3-fold decrease of the minimum detectable concentration for *in vitro* exposure of human blood. Unfortunately, analysis of samples using this technique were often irreproducible, indicating its poor usefulness for an SOP.

Consequently, the SOP for GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine residue in hemoglobin consists of work-up of 20 mg of globin employing the simplified version of the modified Edman degradation, purification of the crude thiohydantoin on a Florisil cartridge, derivatization with heptafluorobutyryl imidazole and, finally, GC-NCI/MS analysis. For detection of lower exposure levels 60 mg of globin can be used, if available. If necessary, the final sample to be analyzed by GC-NCI/MS can be concentrated from 100 to 30 µl, which will result in a more pronounced peak in the chromatogram. In experiments on the day-to-day variability, the adduct levels in human blood exposed to 5 µM sulfur mustard were obtained with a rather large inaccuracy by using this procedure. In the course of the studies performed afterwards it turned out that this inaccuracy was at least partly due to the GC-MS apparatus used which did not function in an optimal way for quantitative analysis.

*Validation of the SOPs*

On the basis of the applied modifications and improvements, SOPs could be drafted which have been validated. The observed intra- and inter-individual variation in N7-HETE-Gua levels determined with the immunoslotblot assay after *in vitro* exposure of human blood to sulfur mustard could be mainly ascribed to the inaccuracy of the procedure (*vide supra*). A standard deviation of 15% of the N7-HETE-Gua level was observed when carrying out the DNA isolation and denaturation on the same day for blood of 10 donors, subsequent to a 5 µM exposure to sulfur mustard.

Unexpected problems arose with respect to the isolation of DNA from blood and from skin after *in vivo* exposure of hairless guinea pigs, as found in parallel studies to this Grant Agreement. The isolation of DNA from blood was complicated by coagulation and precipitation of the blood after freezing/thawing which may be due to heparinization of the animals before administration of sulfur mustard. The problems could be mainly circumvented by performing the experiments without heparinization of the animals. Nevertheless, insufficient amounts were isolated in some cases. For that reason, blood samples were collected by heart puncture in later experiments and were divided in several aliquots, permitting a new attempt to isolate DNA when the first one failed. The problems with DNA isolation from the skin might be due to the extremely thick horny layer of the hairless guinea pig in combination with a thin epidermal layer. As a result, DNA may be lost during isolation due to co-precipitation in the protein precipitation step. Possibly, the horny layer can be pre-separated from the cell suspension by filtration of the cells before lysis. The presence of the thick horny layer may also have a protective effect against the induction of N7-HETE-Gua in DNA of the epidermal cells. This phenomenon has to be taken into account when extrapolating results from validation experiments performed in hairless guinea pigs to human beings.

Administration of 0.1 LD50 or 0.5 LD50 of sulfur mustard (i.v.) to guinea pigs resulted in a dose dependent increase of N7-HETE-Gua in DNA of the white blood cells at 1 h after administration. There was no significant difference in adduct level between 0.1 and 0.5 LD50 at 10 min after administration of sulfur mustard. The adduct level still increased somewhat in the period between 10 min and 1 h after administration of 0.5 LD50, whereas the maximum adduct level was reached at an earlier time point after 0.1 LD50 than after 0.5 LD50.

The persistence studies revealed that during the first two weeks after sulfur mustard administration (0.5 LD50, i.v.) to hairless guinea pigs, the DNA adduct level in blood remains constant, whereas during the following two weeks it decreases to background level. The DNA adduct level after an 8 min exposure of the skin of hairless guinea pigs to saturated sulfur mustard vapor was not significantly different from 10 min up to 1 day after exposure. At two to three days after exposure it had decreased 2- to 3-fold and after 2 weeks the adduct level was only marginal but still detectable. At 6 weeks after exposure no significant adduct levels could be detected. The DNA adduct level in the blood of the marmoset after i.v. administration of sulfur mustard (4.1 mg/kg body weight) was in the same range as in the blood of the guinea pig but decreased much faster than in the guinea pig. After 1 day only marginal levels were observed.

It appeared possible to set up the entire SOP for immunochemical determination of N7-HETE-Gua within one half working day in at MRICD and to generate data in the next one and a half working days on sulfur mustard exposed human blood, which were in agreement with those obtained at TNO-PML.

With respect to validation of the SOP for determination of the alkylated N-terminal valine residue in hemoglobin from human blood , the intra- and interindividual variation of the assay is within the variation due to the inaccuracy of the method (*vide supra*).

The amount of N-terminal valine adduct in globin of hairless guinea pigs which had been exposed to sulfur mustard increases ca. 3-fold when the dose is increased from 0.1 to 0.5 LD50 (i.v.). Furthermore, it was demonstrated that in case of 0.5 LD50 the adduct level 1 h after administration is significantly higher than after 10 min. In case of 0.1 LD50 this effect was less obvious. Care has to be taken with interpretation of these results since single animals were exposed for each experiment.

The persistence studies clearly demonstrated that the N-terminal valine adduct in globin is much more persistent than the DNA adduct, as was to be expected. In blood of hairless guinea pigs, after administration of sulfur mustard (0.5 LD50, i.v.), the globin adduct is persistent for at least 56 days. The adduct level seems to increase in the course of the first day after administration of sulfur mustard and remains more or less stable during the first 28 days. Care has to be taken with further interpretation of these results since for each time point on the curve, a separate animal was exposed. After 56 days, the adduct level has significantly decreased, as can be expected in view of the life time (80-90 days) of the erythrocyte of the (hairless) guinea pig (Dittmer, 1972).

The results obtained with the marmoset are less difficult to interpret. In this case a single animal was followed in time, after administration of sulfur mustard. The results suggest that initially the adduct level increases, as was (probably) also the case for the exposed hairless guinea pigs. This implicates that during this initial time (probably several days) free sulfur mustard is present in the bloodstream and in tissue, which causes accumulating damage over this period of time. Further studies on this interesting phenomenon have been proposed as part of a proposal for a Cooperative Agreement (Langenberg *et al.*, 2000). After a certain period of time (between 1-7 days) the adduct level decreases more or less linearly, in accordance with the life time of the erythrocyte of the animal (Fennell *et al.*, 1992). To the best of our knowledge, the exact life time of erythrocytes in marmosets is not known. After 70 days, the same animal was again challenged with sulfur mustard (4.1 mg/kg, i.v) and blood samples were collected. The experiments gave similar results, albeit that the absolute adduct level in the second experiment was slightly lower, for unknown reasons. The adduct could be analyzed even 94

days after administration of the second dose of sulfur mustard. Comparison with the results obtained for the hairless guinea pigs shows that in marmoset blood adduct levels are approximately one order of magnitude higher, at the same absolute dose. Interestingly, these results are in agreement with toxicokinetic experiments performed by Langenberg *et al.* (1998) under Cooperative Agreement No. DAMD17-94-V-4009, in which much higher levels of intact sulfur mustard were found in blood of marmosets than in blood of guinea pigs, after i.v. administration of of the same absolute dose of sulfur mustard. It can be envisaged that higher levels of the intact agent will lead to higher adduct levels.

The SOP for analysis of sulfur mustard adducts to N-terminal valine of hemoglobin could be properly set up and carried out at MRICD within one working day. The reproducibility and the sensitivity of the assay were similar to those achieved at TNO-PML when using the properly functioning GC-MS equipment.

Upon comparison of the two SOPs it is concluded that

(i) the immunochemical assay for DNA adducts is more suitable for application in a field laboratory and

(ii) can be applied to other samples than blood, e.g., skin biopsies,

whereas

(iii) the modified Edman procedure for determination of adducts to the N-terminal valine in hemoglobin enables detection of exposure of human blood to sulfur mustard within a substantially shorter period of time at the same sensitivity or within the same period of time at a one order of magnitude higher sensitivity and

(iv) enables detection of adducts in blood during a much longer period of time (approximately one order of magnitude) after *in vivo* exposure to sulfur mustard.

**Further exploratory research on immunochemical and mass spectrometric analysis of protein adducts**

The main advantage of detection of adducts to proteins over those to DNA is the expected much longer half-life of the protein adducts. Therefore, antibodies were raised against S-HETE-cysteine in partial sequences of human hemoglobin in our previous studies (Benschop and Van der Schans, 1995). However, the minimum detectable concentration obtained for *in vitro* exposure of human blood with these antibodies was only 50 µM of sulfur mustard. Consequently, further exploratory research on immunochemical assays of protein adducts is the second major topic of the present grant, in addition to the development of SOPs.

Information on immunochemical assays for the detection of protein adducts is only scarcely available in literature. In general, the antibodies have been generated by using adducted keyhole limpet hemocyanin or albumin as an immunogen. These immunogens were obtained by a direct coupling of the reactive compound to the protein, *e.g.*, for 7β,8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (Santella *et al.*, 1986), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Talbot *et al.*, 1990), diclofenac (Pumford *et al.*, 1993), and 4-hydroxy-2-nonenal (Uchida *et al.*, 1994), or by coupling to the immunogen of the adducted amino acid corresponding to the adducted site in the protein, *e.g.*, 3-(N-acetyl-cystein-S-yl)-acetaminophen (Hinson *et al.*, 1995). Such an approach was followed in our previous study (Benschop and Van der Schans, 1995) by using sulfur mustard treated keyhole limpet hemocyanin and hemoglobin as immunogens. However, these experiments did not result in antibodies recognizing adducts in sulfur mustard-treated hemoglobin.

In a more systematic approach haptens having sequential similarity to parts of the adducted protein surface were used for raising antibodies. This approach was only followed in a few studies. Lin *et al.* (1993) generated antibodies against two partial sequences of hemoglobin both adducted with acetaldehyde at lysine residues. Wraith et al. (1988) used the N-terminal heptapeptide of α-globin hydroxyethylated at the terminal amino group as a hapten for raising antibodies against hemoglobin exposed to ethylene oxide. An immunoassay based on the antibody obtained and GC-MS analysis following modified Edman degradation showed comparable results and sensitivities.

A similar approach was followed in the present study. Based on mass spectrometric identification, partial sequences of an adducted protein were synthesized as haptens. Moreover, the results obtained in the mass spectrometric analyses also provide guidance as to which amino acids should be used for quantitative GC-MS or LC-tandem MS analysis, in order to verify immunochemical assays. Investigations were performed on three proteins, *i.e.*, hemoglobin, albumin, and keratin. The accessibility of the adducts for immunochemical analysis is supposed to increase in this order, *i.e.*, hemoglobin is enclosed in erythrocytes, albumin is freely circulating in the plasma, whereas keratins in the skin are directly accessible from the environment for sulfur mustard and for reagents.

**Immunochemical analysis of hemoglobin adducts**

*Characterization of monoclonal antibodies against cysteine-sulfur mustard adducts in hemoglobin*

Antibodies (clone 3H6) raised against an alkylated peptide, *i.e.*, N-acetyl-S-HETE-$cys_{93}$ through $leu_{106}$-lys of the β-chain of hemoglobin, were further characterized. It appeared that exposure of human hemoglobin to 50 μM sulfur mustard was detectable in a direct ELISA. However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have now attempted to apply these antibodies in an immunoslotblot assay to alkylated hemoglobin, but without lowering the minimum detectable concentration. Several other clones were obtained from the above-mentioned immunization which produced antibodies that recognize alkylated hemoglobin.

*Antibodies against peptide haptens containing a histidine-sulfur mustard adduct*

N1/N3-HETE-Histidine is the most abundant amino acid adduct formed in hemoglobin (and albumin, *vide infra*) after exposure of human blood to sulfur mustard (Benschop and Van der Schans, 1995). In addition, three out of the five sites of alkylation within the tertiary structure of hemoglobin are histidine residues, *i.e.*, α-$his_{20}$, β-$his_{77}$ and β-$his_{97}$ (Noort *et al.*, 1996a). Therefore, partial sequences of hemoglobin containing these adducted amino acids were synthesized as haptens for raising antibodies.

In our previous studies (Benschop and Van der Schans, 1995), synthesis of N1/N3-HETE-histidine-containing peptides on a solid support was not successful when using the adducted amino acid unprotected at the 2-hydroxyethylthioethyl group. We have now synthesized a properly protected building block starting from Nα-Boc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine methyl ester, which was obtained by using the semi-mustard derivative 2-(2-*tert*-butyloxyethylthio)ethyl chloride for introduction of a protected 2-hydroxyethylthioethyl group (Benschop and Van der Schans, 1995). The three partial sequences of hemoglobin could conveniently be synthesized on a solid support by using this building block.

We immunized mice with these peptide haptens. From all haptens clones were obtained producing antibodies with specificity for hemoglobin treated with 50 μM sulfur mustard. In the

same experiment, the control clone, 3H6, appeared to be negative which suggests that the test system, particularly the coating of the microtiter plates, was not optimal. Nevertheless, several other clones were positive, suggesting that these clones produced antibodies which were more specific than those from 3H6.

**Mass spectrometric and immunochemical analysis of albumin adducts**

An electrophilic compound has to cross the cell membrane of the erythrocyte in order to react with hemoglobin. Therefore, adduct formation with plasma proteins might be more efficient (Skipper and Tannenbaum, 1990). The most abundant plasma protein is albumin, which has a relatively slow turn-over in human beings (half-life 20-25 days). Covalent binding to albumin of ultimate carcinogens derived from various compounds has been documented, *e.g.*, benzene (Bechtold *et al.*, 1992), benz(a)pyrene (Day *et al.*, 1992), 2-amino-3-methyl-imidazole[4,5-f]quinoline (Lynch *et al.*, 1993), and aflatoxin B1 (Sheabar *et al.*, 1993).

In a first series of experiments, the extent to which albumin is alkylated by sulfur mustard was investigated by using $^{14}C$-labeled agent. It was found that a proportional amount of sulfur mustard (ca. 20%) was bound to albumin isolated from human blood treated with various concentrations (1.3 μM - 1.3 mM) of the agent, indicating a linear relationship between exposure concentration and adduct level. This linear relationship could be further extended down to 10 nM as followed from results obtained in additional experiments in which an alkylated tripeptide in a pronase digest of albumin was analyzed (*vide infra*). Similar results were previously obtained for binding of sulfur mustard (0.1 μM – 5 mM) to hemoglobin (Benschop and Van der Schans, 1995). Although the latter protein is enclosed in erythrocytes, it binds an even somewhat greater fraction of the agent (ca. 25%). These *in vitro* experiments show that binding to albumin and hemoglobin accounts for almost 50% of the total elimination of sulfur mustard, when introduced into human blood.

In order to identify adducted amino acids, both an acidic hydrolysate and tryptic digests of albumin isolated from blood that had been exposed to [$^{14}C$]sulfur mustard were analyzed by means of HPLC after derivatization with Fmoc-Cl. Nα-Fmoc-(N1/N3-HETE)histidine was identified in the acidic hydrolysate from coelution with the synthetic product as the major adducted amino acid, as was also the case for human hemoglobin. The peak of this adducted amino acid accounted for 28% of the total radioactivity bound to the protein. In spite of the abundance of the histidine adduct, it was not attempted to work out a procedure for determination of exposure to sulfur mustard based on this adduct since it cannot be analyzed, up to now, by GC-MS or LC-MS in a sensitive way. The analyses of the tryptic digests showed one major radioactive fragment containing 4-5% of the radioactivity, which was fully separated from other peptide fragments. Therefore, we have not extensively analyzed sulfur mustard alkylation in albumin, but have focused our attention on the major fragment. This fragment was fully identified by LC-tandem MS analysis of the tryptic digest as the T5 peptide of albumin alkylated at cysteine-34. In addition, this alkylated heneicosapeptide synthesized on a solid support coeluted with the major fragment upon HPLC analysis.

In a first series of experiments on quantitative determination of the alkylated peptide, the minimum detectable concentration for *in vitro* exposure of human blood to sulfur mustard was found to be 1 μM by LC-tandem MS analysis under MRM conditions of the tryptic digest, which was not further worked up. Enhancement of the sensitivity was hampered by small signals observed in the blank samples at the same retention time as the analyte. Some of the analyses were performed in the U.K. by using a Q-Tof-mass spectrometer. Since this technique allows to acquire a full scan tandem-MS spectrum of the peptide analyte at the same absolute

sensitivity as provided by an electrospray tandem-MS analysis under MRM conditions, its sensitivity for analysis in biological samples may be enhanced due to a higher potential specificity. However, the minimum detectable concentration could not be improved due to interfering small signals still present in blank samples.

Since selective modification of the alkylated T5 peptide might lead to a clean blank, the peptide was oxidized resulting in the rapid and quantitative formation of the corresponding di-sulfoxide. Unfortunately, this compound did not allow sensitive mass spectrometric identification.

In a second approach to develop an analysis procedure based on the alkylated T5 fragment, the peptide was further digested with pronase, which led to the formation of a di-, tri- and tetrapeptide, all containing the alkylated cysteine residue. These peptides are also formed after direct digestion of adducted albumin by pronase, although the tetrapeptide is formed then only to a very small extent. On the basis of the alkylated di- and tripeptide it was derived that ca. 10% of the total adducts formed in albumin by exposure to sulfur mustard pertains to alkylated cysteine-34. The high sensitivity of cysteine-34 towards alkylation by sulfur mustard is in agreement with previous findings with regard to this amino acid residue in albumin as a nucleophilic site capable of reacting with electrophiles (Bechtold *et al.*, 1992). It is the only reactive sulfhydryl group in the protein. The somewhat lower percentage determined for the alkylated T5 fragment after tryptic cleavage (4-5%) is probably due to incomplete digestion of the protein. Although the percentage of adduct formed with cysteine-34 is less than that of the histidine adducts (28%), it should be kept in mind that the latter adducts can be formed in principle with not less than 16 different residues in the protein.

The most abundant fragment, *i.e.*, the tripeptide (S-HETE)Cys-Pro-Phe, has excellent properties for sensitive mass spectrometric identification. Analyses based on the detection of this alkylated tripeptide in a pronase digest of albumin isolated from Iranian blood samples confirmed the results obtained from analyses with the modified Edman degradation procedure (*vide supra*). The enzymatic degradation of adducted albumin, the work-up and the LC-tandem MS analysis were optimized, resulting in a simple, rapid, reliable and extremely sensitive method. *Using only 3 mg of albumin, we were able to detect exposure to 10 nM of sulfur mustard by applying this method! Presently, this is by far the most sensitive method for detection of exposure of human blood to sulfur mustard.*

We also attempted to raise antibodies against the synthesized T5 fragment (*vide supra*) of human albumin containing an alkylated cysteine. Unfortunately, all originally positive clones lost their specificity for alkylated human albumin during the selection process. Only in six cases stable clones could be obtained with some specificity for the alkylated peptide hapten in comparison to the non-alkylated peptide. These data suggest that the immunogenicity of the HETE-moiety as present in this peptide is not very high and that possibly the alkylated T5-sequence in the sulfur mustard-exposed albumin is poorly accessible.
Antibodies raised against the other alkylated proteins (hemoglobin and keratin) did not show any cross-reactivity with sulfur mustard adducts to albumin.

Finally, the feasibility of the modified Edman degradation on alkylated albumin was investigated. It was investigated whether the amino function of the N-terminal aspartic acid residue in human albumin is alkylated by sulfur mustard. To this end the adduct was synthesized and its derivatization with the modified Edman reagent was attempted. No volatile derivative could be obtained. Consequently, no evidence is obtained for N-alkylation of the

terminal aspartic acid residue in human serum albumin. However, even if formed, it is not suitable for GC/MS analysis after modified Edman degradation.

**Immunochemical and mass spectrometric analysis of keratin adducts**

In addition to the respiratory tract, the skin is a major target for vesicants such as sulfur mustard. Proteins in the skin, particularly those in the stratum corneum, are readily accessible to agents. Since keratin is the most abundant protein in stratum corneum and epidermis, methods for retrospective detection of skin exposure to sulfur mustard were developed in the present study by analyzing adducts formed with this protein.

Keratins (MW 40-70 kDa) form the backbone of the intermediate filaments (IFs) in epithelial tissues (Albers and Fuchs, 1992; Fuchs and Weber, 1994). In basal epidermal cells almost 30% of all synthesized proteins are keratins. Their structures are closely related and can be represented by a central α-helix rich domain (length 300-350 residues) flanked on either side by non-helical domains of variable size and chemical character. The helical segments contain heptad repeats of hydrophobic residues. In addition, a conserved periodic distribution of acidic (aspartic acid, glutamic acid) and basic (arginine, histidine and lysine) amino acids is found in IFs. The termini contain *inter alia* inexact repeats of glycine and (phospho)serine residues. Amino acid sequences of a number of human keratins have been documented [see for instance Albers and Fuchs (1992)]. Most data indicate that the end domains are predominantly located on the surface of the IFs.

In the first series of experiments, the binding to keratin was quantitated and attempts were made to identify adducted amino acids formed in keratin. The experiments were performed with human callus as a model for human skin, which was suspended in a solution of [$^{14}$C]sulfur mustard. The amount of the agent bound to keratin (15-20%) was proportional to the concentration used of sulfur mustard (0.1 μM - 10 mM), as was found for hemoglobin and albumin after exposure of human blood to the agent. Identification of the adducted amino acids was hampered by the absence of radioactively labeled amino acids or peptides in HPLC analyses of the lower molecular fraction obtained by filtration (cut-off 10 kDa) of incubation mixtures of exposed keratin with various proteases, *i.e.*, trypsin, α-chymotrypsin and V8 protease.

It was found that ca. 25% of the radioactivity was split off upon gel filtration of exposed keratin at pH 7.6, whereas not less than 80% of total radioactivity eluted as material with low molecular mass by gel filtration of exposed keratin that was treated with aqueous NaOH (0.5 M). This product was identified as thiodiglycol. Our results suggest strongly that most of the adducts formed with keratin are esters of thiodiglycol with glutamic and aspartic acid residues, which are readily hydrolyzed with base. Therefore, we focused our attention on analysis of sulfur mustard adducts with these two amino acid residues for the development of both an immunochemical and a mass spectrometric method for retrospective detection of exposure of skin to sulfur mustard.

For development of an immunochemical assay, two partial end domain sequences of keratin K14 and one partial end domain sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies. The peptides contain adducted glutamine or asparagine instead of the corresponding thiodiglycol esters with glutamic acid and aspartic acid. The resulting amides are expected to be stable during immunization in contrast to the thiodiglycol esters. Initially, it was attempted to obtain the adducted amino acid residues on a solid support after selective removal of a protecting allyl function from the glutamine or asparagine residue when

the peptide was still attached to the solid support. However, we did not succeed in splitting off the protecting group at these conditions. We then synthesized properly protected building blocks for the two adducted amino acids, analogously to the approach followed for the synthesis of N1/N3-HETE-histidine containing peptides on a solid support (*vide supra*). Also in this case, the desired peptides could be synthesized conveniently by using these synthons.

This approach appeared to be very successful for raising antibodies. Fusions from mice immunized with only one peptide or with a mixture of two or three peptides resulted in specific antibodies to sulfur mustard adducts in keratin isolated from human callus. Moreover, some of the monoclonal antibodies clearly showed binding to the horny layer of human skin exposed to a solution of 50 or 100 µM sulfur mustard. An even more pronounced effect was observed when the skin had been exposed to saturated vapor of sulfur mustard for 1 min (Ct ca. 1040 $mg.min.m^{-3}$). It should be emphasized that the antibodies were directly applied to human skin samples without pre-conditioning of the sample. *This opens the way for development of a detection kit that can be applied directly to the skin of personnel who are possibly exposed to sulfur mustard.*

Mass spectrometric analysis of thiodiglycol that is released by mild base from keratin exposed to sulfur mustard should be an attractive method for retrospective detection of skin exposure. The analyte is obtained in a simple way without degradation of the protein, avoiding generation of other low molecular material. In addition, the ready release of thiodiglycol from sulfur mustard exposed keratin opens the way for direct detection of adducts in the skin without taking biopsies, by treating the skin with appropriate reagents. In experiments to develop a mass spectrometric method, it was demonstrated that thiodiglycol could efficiently be isolated by filtration and subsequent concentration after alkaline treatment at pH 13 of keratin that was exposed to sulfur mustard. After derivatization with pentafluorobenzoyl chloride according to a procedure reported in literature (Black and Read, 1988), the thiodiglycol derivative could be analyzed by GC-NCI/MS with a detection limit of 5 pg. This procedure allowed detection of exposure of human callus to 10 µM of sulfur mustard. The same procedure could be applied to keratin extracts of human skin that was exposed to saturated sulfur mustard vapor *in vitro*. A linear relationship was observed between the amount of released thiodiglycol and exposure time. It is envisaged that this method can be used to verify the results obtained by the more rapid immunochemical method for detection of keratin - sulfur mustard adducts described above.

In order to enable direct detection of sulfur mustard adducts in the skin, the hydrolysis of the thiodiglycol esters in keratin should preferably be performed at lower pH. Unfortunately, substantial release of thiodiglycol was not found at pH ≤ 11. Addition of compounds, such as histidine and urea, in order to accelerate hydrolysis at pH 9 or treatment of keratin with an aqueous solution of various primary alkylamines did not result in a significant increase of the amount of thiodiglycol. Enzymatic hydrolysis was not successful either.

**Cross-reactivity of antibodies for sulfur mustard adducts with hemoglobin, albumin and keratin**

Cross-reactivity was observed for antibodies raised against partial sequences of hemoglobin containing a histidine-sulfur mustard adduct. These antibodies show specificity not only for alkylated hemoglobin but also for alkylated keratin. This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound. If so, these antibodies can be valuable for immunochemical staining applications, *e.g.*, in histochemical identification of alkylated cell proteins in human skin.

These antibodies did not show specificity to albumin or trypsinized albumin alkylated by sulfur mustard. Similar negative results were obtained for one of the clones raised against a partial sequence of keratin containing a glutamine-sulfur mustard adduct, which showed a positive response on sulfur mustard-exposed human skin. We were also unsuccessful in raising antibodies against sulfur mustard treated albumin when using a partial sequence of albumin containing the major adduct formed in this protein with sulfur mustard (cysteine-34, *vide supra*). These results may suggest that sulfur mustard adducts in albumin are poorly accessible.

In a parallel study to this Grant Agreement, we examined the alkylation of phosphoserine residues by sulfur mustard in keratin from human epidermis. It has been reported that phosphate mono-esters exhibit high reactivity towards mustard agents (Davis and Ross, 1952). The termini of keratins contain (inexact) repeats of glycine and (phospho)serine residues. Two peptides both containing a phosphoserine-sulfur mustard adduct were synthesized (Noort *et al.*, 1996b) and served as haptens for raising antibodies. Several clones have been obtained producing antibodies not only specific for alkylated (phosphoserine-containing) keratin but also for alkylated hemoglobin. Some clones produced antibodies which were even more specific for alkylated hemoglobin than for alkylated keratin. This suggests once more that the specificity depends in some cases mainly on the presence of the adduct rather than on the amino acid to which the adduct is bound.

# VI KEY RESEARCH ACCOMPLISHMENTS

1. A convenient route for synthesis of [$^{14}$C]sulfur mustard was developed, which leads to higher and more reproducible yields than the route for synthesis of the $^{35}$S-labeled agent.

2. The first steps of an immunoslotblot assay of sulfur mustard adducts to DNA in human blood and skin, *i.e.*, DNA isolation, could be shortened in labor time from one and a half day and two overnight incubations to 3 h labor time and one overnight incubation. The total DNA isolation time could be even further reduced to about 4-5 h when performed with fresh blood.

3. A significant reduction in time (ca. a half working day) and labor for performing an immunoslotblot assay was achieved by using a luminometer for measurement of the chemiluminescence instead of the combination of a photographic film and a densitometer for measurement of the blackening. Altogether, the total assay time, including the DNA isolation procedure, takes about 1.5 working days and two overnight incubation steps.

4. The lower detection limit of the immunoslotblot assay was reached at 8-40 amol N7-HETE-Gua/blot using 1 µg DNA, corresponding to an adduct level of 3-13 N7-HETE-Gua/$10^9$ nucleotides. The minimum detectable concentration for *in vitro* treatment of human blood with sulfur mustard was 50 nM.

5. Although there is a substantial day-to-day variation due to irregularities during DNA isolation and denaturation, the inter-individual variation in adduct level after *in vitro* exposure of human blood to 5 µM sulfur mustard is only ca. 15%.

6. *In vivo* persistence studies in hairless guinea pigs revealed that N7-HETE-Gua is detectable in blood during the first two weeks after administration of a dose of sulfur mustard corresponding to 0.5 LD50 (i.v.). In skin the DNA-adduct level decreases only slowly during the first 3 days and is marginal at 17 days after exposure.

7. An SOP for immunoslotblot assay of N7-HETE-Gua in DNA of white blood cells and in skin biopsies has been drafted.

8. It appeared possible to set up the complete instrumentation for the immunoslotblot assay within a half working day at MRICD and to generate data within the next one and a half day, including the overnight incubation with 1st antibody, using DNA samples isolated previously from sulfur mustard exposed blood. These data were in agreement with those obtained at TNO-PML.

9. According to a shortened SOP, N7-HETE-Gua in DNA of white blood cells can be detected within 9 h after *in vitro* exposure of human blood to ≥ 1 µM sulfur mustard.

10. The modified Edman procedure for determination of sulfur mustard adducts to the N-terminal valine in hemoglobin including GC-NCI/MS analysis can be shortened to one working day without loosing sensitivity, by performing the Edman degradation reaction for 2 h at 60 °C instead of overnight at room temperature followed by 2 h at 45 °C.

11. A substantial purification of the crude thiohydantoin was achieved by introducing a solid phase extraction step into the modified Edman procedure, which allows to process larger amounts of globin up to 60 mg, although this does not result in a significant decrease of the minimum detectable concentration, *i.e.*, 100 nM.

12. Application of a TCT injection technique in the GC-NCI/MS analysis of the final sample obtained after the modified Edman procedure led to a 3-fold lower minimum detectable concentration for *in vitro* exposure of human blood, but is insufficiently reproducible.

13. An SOP for the modified Edman procedure has been drafted.

14. The day-to-day variability in the adduct level determined by using the modified Edman procedure was reasonable.

15. The intra-individual variability in adduct levels determined by using the modified Edman procedure was reasonable.

16. The inter-individual variability in adduct levels determined by using the modified Edman procedure was reasonable.

17. The amount of N-terminal valine adduct in globin of hairless guinea pigs after administration of doses of sulfur mustard corresponding with 0.1 and 0.5 LD50 (i.v.) increases with the dose.

18. The N-terminal valine adduct in globin of hairless guinea pigs can be analyzed for at least 56 days after administration of sulfur mustard (0.5 LD50, i.v.)

19. The N-terminal valine adduct in globin of a marmoset can be analyzed for at least 94 days after administration of sulfur mustard (4.1 mg/kg).

20. The level of N-terminal valine adduct in blood of marmosets is one order of magnitude higher than in blood of hairless guinea pigs, after i.v. administration of the same absolute dose (4.1 mg/kg) of sulfur mustard to both species.

21. The SOP for analysis of sulfur mustard adducts to N-terminal valine of hemoglobin could be properly set up and carried out at MRICD within 9 h.

22. A convenient route has been worked out for synthesis of a properly protected building block of N1/N3-HETE-histidine that is suitable for solid phase synthesis of peptides.

23. Partial sequences of hemoglobin containing an adduct with a histidine that was previously identified as an alkylation site for sulfur mustard, *i.e.*, $\alpha$-his$_{20}$, $\beta$-his$_{77}$ or $\beta$-his$_{97}$, were synthesized as haptens for raising antibodies against sulfur mustard treated hemoglobin.

24. Several clones are available producing antibodies which show specificity not only for hemoglobin alkylated with 50 µM sulfur mustard but also for alkylated keratin.

25. A proportional amount of [$^{14}$C]sulfur mustard (ca. 20%) was bound to albumin isolated from human blood treated with various concentrations of the agent.

26. (N1/N3-HETE)Histidine was identified in an acidic hydrolysate of albumin isolated from human blood that had been exposed to sulfur mustard, accounting for 28% of the total amount of adducts formed.

27. HPLC analysis of tryptic digests of albumin isolated from human blood that had been exposed to [$^{14}$C]sulfur mustard showed one major fragment which was fully separated from other peptide fragments and was fully identified by LC-tandem MS analysis as the T5 fragment of albumin alkylated at cysteine-34.

28. Approximately 10% of the total adducts formed in albumin by exposure of human blood to sulfur mustard pertain to alkylated cysteine-34.

29. The alkylated T5 peptide of human albumin was synthesized on a solid support as a hapten for raising antibodies against sulfur mustard treated albumin. Unfortunately, the antibodies raised against this peptide did not recognize adducts with the alkylated albumin, suggesting a poor accessibility.

30. LC-tandem MS analysis under MRM conditions performed directly on the T5 fragment in a tryptic digest of albumin that was isolated from sulfur mustard treated human blood allowed to detect exposure to ≥ 1 μM of the agent.

31. Exposure of human blood to ≥ 10 nM sulfur mustard can be detected by a simple, rapid and reliable method based on LC-tandem MS analysis of the tripeptide (S-HETE)Cys-Pro-Phe in a pronase digest of only 3 mg albumin. *Presently, this is by far the most sensitive method for detection of exposure of human blood to sulfur mustard.*

32. LC-tandem MS analyses of the tripeptide (S-HETE)Cys-Pro-Phe in a pronase digest of albumin in 12 years old blood samples taken from Iranian victims of the Iran-Iraq conflict confirmed exposure to sulfur mustard as found from analyses by means of the modified Edman procedure of the N-terminal valine of hemoglobin in the same samples.

33. The amount of [$^{14}$C]sulfur mustard bound to keratin in human callus (15-20%) was proportional to the concentration of the agent in the solution in which human callus had been suspended.

34. Most of the adducts formed with keratin in human callus that was exposed to [$^{14}$C]sulfur mustard are esters of thiodiglycol with glutamic and aspartic acid residues, since treatment of exposed keratin with aqueous NaOH (0.5 M) released 80% of the total radioactivity, which was identified as thiodiglycol.

35. Treatment at pH ≥ 13 is necessary to achieve substantial release of thiodiglycol from keratin that had been exposed to sulfur mustard, which is probably unacceptable for *in vivo* application.

36. GC-NCI/MS of thiodiglycol that was released by alkaline treatment from keratin, isolated from human callus exposed to sulfur mustard *in vitro* and derivatized with pentafluorobenzoyl chloride, allows detection of exposure of human callus to ≥ 10 μM sulfur mustard.

37. Exposure of human skin to ≥ 2 min of saturated sulfur mustard vapor can be detected by GC-NCI/MS analysis of thiodiglycol released by alkaline treatment of keratin extracts, after derivatization with pentafluorobenzoyl chloride.

38. Two partial end domain sequences of keratin K14 and one partial end domain sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies, by using a properly protected building block of glutamine or asparagine adducted with a 2-hydroxyethylthioethyl group at the amide function.

39. Several clones raised against the haptens as mentioned in item 38 are available producing antibodies which show specificity for keratin isolated from human callus alkylated with 50 μM sulfur mustard.

40. Some of the clones mentioned in item 39 clearly showed binding to the horny layer of human skin exposed to a solution of 50 μM sulfur mustard, whereas an even more pronounced effect was observed when human skin had been exposed to saturated vapor of sulfur mustard for 1 min (Ct ca. 1040 mg.min.m$^{-3}$).

41. In the experiments mentioned in conclusion 41, the antibodies were directly applied to the human skin cross-sections without extreme pre-conditioning of the skin. *This opens the way for development of a detection kit that can be applied directly to the skin of human beings which has possibly been exposed to sulfur mustard.*

## VII REPORTED OUTCOMES

**Publications**

NOORT, D., HULST, A.G., DE JONG, L.P.A., AND BENSCHOP, H.P. (1999) Alkylation of human serum albumin by sulfur mustard *in vitro* and *in vivo*: mass spectrometric analysis of a cysteine adduct as a sensitive biomarker of exposure.
Chem. Res. Toxicol. **12**, 715-721.

FIDDER, A., NOORT, D., AND BENSCHOP, H.P. (1999) A convenient synthesis of [$^{14}$C]1,1'-thiobis(2-chloroethane), [$^{14}$C]sulfur mustard.
J. Lab. Comp. Radiopharm. **42**, 261-266.

BENSCHOP, H.P., NOORT, D., AND BLACK, R.M. (1999) Methods for retrospective detection of exposure to toxic scheduled chemicals: an overview. UK/NL Non-Paper to the OPCW; 6 December 1999.

**Abstracts**

NOORT, D. (1997) Mass spectrometric detection of sulfur mustard adducts to DNA and hemoglobin.
Abstract book "5th European Meeting on Mass Spectrometry in Occupational and Environmental Health" (9-11 June, Nijmegen, The Netherlands), pp 63-64.

NOORT, D., FIDDER, A., DE JONG, L.P.A., AND BENSCHOP, H.P. (1998) Diagnosis and dosimetry of exposure to sulfur mustard: development of standard operating procedures for hemoglobin adducts; exploratory research on albumin and keratin adducts.
Program Book Bioscience Review (31 May - 4 June, 1998, Hunt Valley, MD), pp 116-117.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., DE JONG, L.P.A., BRUIJNZEEL, P.L.B., AND BENSCHOP, H.P. (1998) Immunochemical detection of sulfur mustard-adducts with DNA and proteins; development of standard operating procedures for DNA adducts and exploratory research on adducts with proteins.
Program Book Bioscience Review (31 May - 4 June, 1998, Hunt Valley, MD), pp 144-145.

NOORT, D. (1999) An improved method for diagnosis of exposure to sulphur mustard: mass spectrometric analysis of adducts to human serum albumin.
Abstract Book "NATO TG 004 Meeting", March 22-26, Brussels.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., DE JONG, L.P.A., BRUIJNZEEL, P.L.B., AND BENSCHOP, H.P. (1999) Immunochemical detection of sulfur mustard-adducts with DNA and proteins; exploratory research on adducts with proteins.
Abstract Book "NATO TG 004 Meeting", March 22-26, Brussels.

**Presentations**

Mass spectrometric detection of sulfur mustard adducts to DNA and hemoglobin.
Presented by D. Noort at "5th European Meeting on Mass Spectrometry in Occupational and Environmental Health", June 9-11, 1997, Nijmegen, The Netherlands.

An improved method for diagnosis of exposure to sulphur mustard: mass spectrometric analysis of adducts to human serum albumin.
Presented by D. Noort at "NATO TG 004 Meeting", March 22-26, 1999, Brussels, Belgium.

Immunochemical detection of sulfur mustard-adducts with DNA and proteins; exploratory research on adducts with proteins.
Presented by G.P. van der Schans at "NATO TG 004 Meeting", March 22-26, 1999, Brussels, Belgium.

Methods for retrospective detection of exposure to toxic scheduled chemicals: an overview.
To be presented at CB-MTS III symposium, May 7-12, 2000, Spiez, Switzerland.

# VIII CONCLUSIONS

Within the framework of previous grants we have developed methods for retrospective detection of exposure to sulfur mustard which are based on the development of monoclonal antibodies against adducts of sulfur mustard with DNA and proteins for use in a variety of immunochemical assays. In addition, LC-tandem MS and GC-MS analyses of the adducts were developed for validation of the immunochemical assays. Two methods had been sufficiently worked out to justify the development of SOPs for application in a well-equipped field hospital, *i.e.*, an immunoslotblot assay of sulfur mustard adducts to DNA in human blood and skin, and a GC-NCI/MS determination of sulfur mustard adducts to the N-terminal valine in hemoglobin of human blood by using the modified Edman procedure. Development of these SOPs is one of the two major topics of the present grant. It was intended to develop two modes of immunoassay SOPs, *i.e.*, one in which experimental time is as short as possible and another one in which sensitivity is the most important factor.

The modifications applied to the immunoslotblot assay were aimed at sim,plification and shortening of the procedure while maintaining maximum sensitivity. The procedure for DNA isolation from human blood for the immunochemical determination of N7-(2-hydroxyethylthioethyl)-guanine (N7-HETE-Gua) in DNA has been shortened from one and a half day labor time and two overnight incubations to ca. 3 h labor time and one overnight incubation, using only 300 µl of blood. The total DNA isolation time could be even reduced to ca. 4-5 h when performed with fresh blood. In addition, the sensitivity of the immunoslotblot assay could be improved by UV crosslinking of the DNA in 1-µg quantities to the nitrocellulose filter and by direct measurement of the chemiluminescence with a luminometer. The lower detection limit is now in the range of 1-13 N7-HETE-Gua/$10^9$ nucleotides and was previously ca. 300 N7-HETE-Gua/$10^9$ nucleotides. By using this modified procedure, treatment of double stranded calf thymus DNA to ≥ 2.5 nM sulfur mustard could be detected. The adduct levels detected with the improved procedure in DNA of blood exposed to sulfur mustard were much lower than expected, for unknown reasons. Nevertheless, a minimum detectable concentration for exposure of human blood *in vitro* to 50 nM sulfur mustard is feasible instead of 70 nM previously. The immunoslotblot assay showed a rather large day-to-day variation, corresponding to an up to 2-fold difference in adduct level, which appeared to be mainly introduced during DNA isolation and denaturation. The observed intra- and inter-individual variation in adduct levels after *in vitro* exposure of human blood to sulfur mustard could be mainly ascribed to that phenomenon. In addition, modifications have been introduced in the assay to further shorten the procedure while accepting some decrease in sensitivity and accuracy. When this shortened procedure was applied, data could be generated within 9 h after *in vitro* exposure of human blood. In this way, exposure of human blood to 1 µM sulfur mustard was still detectable.

Unexpected problems arose with respect to the isolation of DNA from blood as well as skin after *in vivo* exposure of hairless guinea pigs, which had consequences for our planned *in vivo* validation studies for the immunoslotblot assay. It appeared to be necessary to take blood samples by heart puncture, after killing the animal. In addition, the observed lower N7-HETE-Gua levels in DNA of the skin of the hairless guinea pig in comparison to those in human skin exposed to the same dose suggested that the thick horny layer of the hairless guinea pig may have a protective effect against the induction of N7-HETE-Gua in DNA of the epidermal cells. This phenomenon has to be taken into account when extrapolating results from validation experiments performed in hairless guinea pigs to human beings. The persistence studies revealed that during the first two weeks after sulfur mustard administration the DNA-adduct level in blood remains constant, whereas during the following two weeks it decreases to background level. The adduct level after exposure of the skin of hairless guinea pigs to

saturated sulfur mustard vapor was not significantly different from 10 min up to 1 day after exposure. At two to three days after exposure it had decreased 2- to 3-fold and after 2 weeks the adduct level was only marginal but still detectable. At 6 weeks after exposure no significant DNA-adduct levels could be detected. The DNA-adduct level in the blood of the marmoset after i.v. administration of sulfur mustard (4.1 mg/kg body weight) was in the same range as in the blood of the guinea pig but decreased more rapidly than in the guinea pig. After 1 day only marginal levels were observed in blood of the marmoset.

It appeared possible to set up the entire SOP for immunochemical detection of N-HETE-Gua within an acceptable period of time (one half working day) in a US Army Institute (USAMRICD) and to generate data within the next one and a half working days on sulfur mustard exposed human blood which were in agreement with those obtained at TNO-PML.

The modified Edman procedure for determination of sulfur mustard adducts to the N-terminal valine in hemoglobin including GC-NCI/MS analysis could be shortened from two working days to one without loosing sensitivity, by performing the Edman degradation reaction for 2 h at 60 °C instead of overnight at room temperature followed by 2 h at 45 °C. The N-terminal valine adduct in hemoglobin was detected with this procedure in blood samples taken from nine Iranian victims of the Iran-Iraq conflict, who were exposed to sulfur mustard 8-9 days earlier. These results could be confirmed by analyses based on the detection of an alkylated tripeptide in a pronase digest of albumin isolated from these blood samples (*vide infra*).

A substantial purification of the crude thiohydantoin was achieved by introducing a solid phase extraction step into the modified Edman procedure, which allows us to process a three-fold larger amount of globin. However, a significant decrease of the detection limit of the procedures was not achieved. Application of the thermodesorption/cold trap (TCT) injection technique in the GC-NCI/MS analysis of the final sample obtained after the modified Edman procedure led to a 3-fold decrease of the minimum detectable concentration (from 0.1 to 0.03 μM) for *in vitro* exposure of human blood. Unfortunately, analyses in which this technique was applied could not routinely be performed, since the results were not sufficiently reproducible. On the basis of these results, an SOP has been drafted. Several experiments were carried out for validation of the SOP. Experiments on the day-to-day variability showed a rather large inaccuracy in the adduct levels obtained with this procedure. In the course of the studies performed afterwards, and at MRICD, it turned out that this inaccuracy was at least partly due to the GC-MS apparatus used which did not function in an optimal way for quantitative analysis. The intra-individual variation and the inter-individual variation in the adduct level in human blood determined by using this SOP were within the variation due to the inaccuracy of the method. Furthermore, it was assessed that the amount of N-alkylated terminal valine in globin of hairless guinea pigs, after administration of sulfur mustard (0.1 and 0.5 LD50, i.v.), increases with the dose. It was found that the N-terminal valine adduct in globin of hairless guinea pigs after administration of sulfur mustard (0.5 LD50, i.v.) is persistent for at least 56 days. In case of the marmoset this is even longer, *i.e.* 94 days. The level of N-terminal valine adduct in blood of marmosets is one order of magnitude higher than in blood of hairless guinea pigs, after i.v. administration of the same absolute dose (4.1 mg/kg) of sulfur mustard to both species. The SOP for analysis of sulfur mustard adducts to N-terminal valine of hemoglobin could be properly set up and carried out at MRICD within one working day.

Upon comparison of the two SOPs it is concluded that

(i) the immunochemical assay for DNA adducts is more suitable for application in a field laboratory and

(ii) can be applied to other samples than blood, e.g., skin biopsies,

whereas

(iii) the modified Edman procedure for determination of adducts to the N-terminal valine in hemoglobin enables detection of exposure of human blood to sulfur mustard within a substantially shorter period of time at the same sensitivity or within the same period of time at a one order of magnitude higher sensitivity and

(iv) enables detection of adducts in blood during a much longer period of time (approximately one order of magnitude) after *in vivo* exposure to sulfur mustard.

The retrospectivity of the diagnosis based on protein adducts is superior to that based on DNA adducts due to the much longer half lives of protein adducts. Therefore, exploratory research was performed aiming at the development of a fieldable sensitive immunochemical assay for sulfur mustard adducts with three proteins, *i.e.*, hemoglobin, albumin, and keratin. In various series of these experiments, [$^{14}$C]sulfur mustard has advantageously been used. A synthetic route was developed for this compound which leads to higher and more reproducible yields than the route for synthesis of the $^{35}$S-labeled agent used in previous studies.

A properly protected building block of N1/N3-HETE-histidine was synthesized. This synthon was used in the solid phase synthesis of three partial sequences of hemoglobin containing an adducted histidine identified as a major alkylation site in the protein. Several clones have been obtained using these three peptides as haptens, in addition to the clone 3H6 which was raised against N-acetyl-S-HETE-cys$_{93}$ through leu$_{106}$-lys of the β-chain of hemoglobin. Antibodies of these clones show specificity not only for hemoglobin alkylated with 50 μM sulfur mustard but also for alkylated keratin. This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound. Consequently, these antibodies might be promising for application in immunostaining in tissues.

Upon exposure of human blood to various concentrations (1.3 μM-1.3 mM) of [$^{14}$C]sulfur mustard we found that a proportional amount (ca. 20%) was covalently bound to albumin. This linear relationship was further extended down to 10 nM by experiments in which an alkylated tripeptide in a pronase digest of albumin was analyzed (*vide infra*). The major adducted amino acid formed by acidic hydrolysis was N1/N3-HETE-histidine (28%). The major fragment formed by tryptic digestion was identified by LC-tandem MS analysis as the T5 peptide alkylated at cysteine-34, *i.e.*, HETE-(A-L-V-L-I-A-F-A-Q-Y-L-Q-Q-C-P-F-E-D-H-V-K). This peptide was synthesized on a solid support as a hapten for raising antibodies against sulfur mustard treated albumin. LC-tandem MS analysis under multiple reaction monitoring conditions performed directly in a tryptic digest of albumin that was isolated from sulfur mustard treated human blood allowed to detect exposure to ≥ 1 μM of the agent. Unfortunately, the minimum detectable concentration could not be decreased due to interfering small signals still present in blank samples, whereas the disulfoxide obtained upon selective modification of the alkylated T5 fragment did not allow sensitive mass spectrometric identification.

Treatment of alkylated albumin with pronase led to the formation of a di- and tripeptide as the main fragments containing alkylated cysteine-34. On the basis of these alkylated peptides it was derived that ca. 10% of the total adducts formed in albumin by exposure to sulfur mustard pertain to alkylated cysteine-34. The tripeptide, (S-HETE)Cys-Pro-Phe, has excellent properties for sensitive mass spectrometric identification. The enzymatic degradation of adducted albumin, the work-up and the LC-tandem MS analysis were optimized, resulting in a simple, rapid, reliable and extremely sensitive method. *Using only 3 mg of albumin, we were able to detect exposure to 10 nM of sulfur mustard by applying this method. Presently, this is by far the most sensitive marker for exposure of human blood to sulfur mustard.* Attempts to apply the modified

Edman procedure for GC-MS determination of N-alkylated aspartic acid, which might be formed upon exposure of albumin to sulfur mustard, were not successful.
Attempts to produce antibodies directed against sulfur mustard adducts to albumin, using the alkylated T5 fragment as hapten, were not successful. Probably, the alkylated Cys34 residue in the hapten used is not enough immunogenic and/or albumin is not sufficiently accessible for the antibodies.

Upon exposure of human callus (suspension in 0.9% NaCl; 20 mg/ml) to various concentrations of [$^{14}$C]sulfur mustard we found that 15-20% of the added radioactivity was covalently bound to keratin. Unfortunately, enzymatic digestion in order to identify specific alkylated sites did not give satisfactory results. Upon incubation with base (pH 13), 80% of the bound radioactivity was split off as [$^{14}$C]thiodiglycol, which suggests that most of the adducts formed with keratin in human callus are esters of thiodiglycol with glutamic and aspartic acid residues. After derivatization of thiodiglycol with pentafluorobenzoyl chloride, the derivative could be analyzed by GC-NCI/MS with a detection limit of 5 pg. This procedure allowed detection of exposure of human callus to $\geq$ 10 μM of sulfur mustard. The same procedure could be used for analysis of thiodiglycol that was released by alkaline treatment from keratin, isolated from human skin which had been exposed to saturated sulfur mustard vapor. Unfortunately, substantial release of thiodiglycol was found neither at pH $\leq$ 11 nor with additives such as histidine and urea at pH 9 or treatment with an aqueous solution of various primary alkylamines. Enzymatic hydrolysis was not successful either. Therefore, the procedure is not yet applicable to human skin *in vivo*.

Two partial sequences of keratin K14 and one partial sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies, by using a properly protected building block of glutamine or asparagine adducted with a 2-hydroxyethylthioethyl group at the ω-amide function. Such amide analogs are presumably more stable *in vivo* than the corresponding esters, which can be substrates for esterase-type enzymes. After immunization, monoclonal antibodies were obtained which are specific to sulfur mustard adducts in keratin isolated from human callus. Moreover, some of the antibodies clearly showed binding to the horny layer of human skin exposed to a solution of 50 μM sulfur mustard. An even more pronounced effect was observed when the skin had been exposed to saturated vapor of sulfur mustard (Ct 1040 mg.min.m$^{-3}$). It should be emphasized that the antibodies were directly applied to human skin samples without pre-conditioning of the sample. *This opens the way for development of a detection kit that can be applied directly to skin of personnel which had possibly been exposed to sulfur mustard.*

For some antibodies directed against sulfur mustard adducts to hemoglobin cross-reactivity was observed with sulfur mustard adducts to keratin, but not with sulfur mustard adducts to albumin.

# IX REFERENCES

ALBERS, K. AND FUCHS, E. (1992) The molecular biology of intermediate filament proteins. Int. Rev. Cytology **134**, 243-279.

BAAN, R.A., SCHOEN, M.A., ZAALBERG, O.B. AND LOHMAN, P.H.M. (1982) The detection of DNA damages by immunochemical techniques. In: Mutagens in Our Environment (Sorsa, M. and Vainio, H., Eds), Alan R Liss, New York, pp. 111-124.

BAILEY, E., FARMER, P.B. AND SHUKER, D.E.G (1987) Estimation of exposure to alkylating carcinogens by the GC-MS determination of adducts to hemoglobin and nucleic acid bases in urine. Arch. Toxicol. **60**, 187-191.

BECHTOLD, W.E., WILLIS, J.K., SUN, J.D., GRIFFITH, W.C. AND REDDY, T.V. (1992) Biological markers of exposure to benzene: S-phenylcysteine in albumin. Carcinogenesis **13**, 1217-1220.

BENSCHOP, H.P. (1991) Verification, dosimetry and biomonitoring of mustard gas exposure via immunochemical detection of mustard gas adducts to DNA and proteins. Final report for Grant DAMD17-88-Z-8022, NTIS AD-A240 471.

BENSCHOP, H.P. AND VAN DER SCHANS, G.P. (1995) Immunochemical and mass spectrometric detection of mustard gas adducts to DNA and proteins: verification and dosimetry of exposure to mustard gas. Final report for Cooperative Agreement DAMD17-92-V-2005.

BENSCHOP, H.P., VAN DER SCHANS, G.P., NOORT, D., FIDDER, A., MARS-GROENENDIJK, R.H. AND DE JONG, L.P.A. (1997) Verification of exposure to sulfur mustard in two casualties of the Iran-Iraq conflict. J. Anal. Toxicol. **21**, 249-251.

BENT, H.E. (1947) The laboratory preparation of mustard gas. Science **106**, 374-375.

BLACK, R.M. AND READ, R.W. (1988) Detection of trace levels of thiodiglycol in blood, plasma and urine using gas chromatography-electron-capture negative-ion chemical ionisation mass spectrometry. J. Chromatogr. **449**, 261-270.

CHO, M., JEDRYCHOWSKI, R., HAMMOCK, B. AND BUCKPITT, A. (1994) Reactive naphtalene metabolite binding to hemoglobin and albumin. Fundam. Appl. Toxicol. **22**, 26-33.

CRODDY, E. (1995) Urban terrorism - chemical warfare in Japan. Jane's Intelligence Review **7**, 520.

DAVIS, W. AND ROSS, W.C.J. (1952) Aryl-2-halogenoalkylamines. Part XI. The reaction of NN-di-2'-chloroethyl-2-naphthylamine with some phosphoric acid derivatives. J. Chem. Soc., 4296-4300.

DAY, B.W., DOXTADER, M.M., RICH, R.H., SKIPPER, P.L., SINGH, K., DASARI, R.R. AND TANNENBAUM, S.R. (1992) Human serum albumin-benzo[*a*]pyrene *anti*-diol epoxide adduct structure elucidation by fluorescence line narrowing spectroscopy. Chem. Res. Toxicol. **5**, 71-76.

DITTMER, D.S., Ed.(1972) Biology Data Book, Vol. I, FASEB, Bethesda, MD.

FENNELL, T.R., SUMNER, S.C.J. AND WALKER, V.E. (1992) A model for the formation and removal of hemoglobin adducts. Cancer Epidemiology, Biomarkers & Prevention **1**, 213-219.

FIDDER, A., MOES, G.W.H., SCHEFFER, A.G., VAN DER SCHANS, G.P., BAAN, R.A., DE JONG, L.P.A. AND BENSCHOP, H.P. (1994) Synthesis, characterization and quantification of the major adducts formed between sulfur mustard and DNA of calf thymus and human blood. Chem. Res. Toxicol. **7**, 199-204.

FIDDER, A., NOORT, D., DE JONG, A.L., TRAP, H.C., DE JONG, L.P.A. AND BENSCHOP, H.P. (1996a) Monitoring of *in vitro* and *in vivo* exposure to sulfur mustard by GC/MS determination of the N-terminal valine adduct in hemoglobin after a modified Edman degradation. Chem. Res. Toxicol. **9**, 788-792.

FIDDER, A., NOORT, D., HULST, A.G., DE JONG, L.P.A. AND BENSCHOP, H.P. (1996b) N7-(2-hydroxyethylthioethyl)-guanine: a novel urinary metabolite following exposure to sulfur mustard. Arch. Toxicol. **70**, 854-855.

FIDDER, A., NOORT, D. AND BENSCHOP, H.P. (1999) A convenient synthesis of [$^{14}$C]1,1'-thiobis(2-chloroethane), [$^{14}$C]sulfur mustard. J. Lab. Comp. Radiopharm. **42**, 261-266.

FUCHS, E. AND GREEN, H. (1978) The expression of keratin genes in epidermis and cultured epidermal cells. Cell **15**, 887-897.

FUCHS, E. AND WEBER, K. (1994) Intermediate filaments: structure, dynamics, function, and disease. Annu. Rev. Biochem. **63**, 345-382.

HINSON, J.A., PUMFORD, N.R. AND ROBERTS, D.W. (1995) Mechanisms of acetaminophen toxicity: immunochemical detection of drug-protein adducts. Drug Metab. Rev. **27**, 73-92.

LANGENBERG, J.P., BENSCHOP, H.P. AND VAN DER SCHANS, G.P. (1998) Toxicokinetics of sulphur mustard and its DNA-adducts in the hairless guinea pig - DNA adducts as a measure for epithelial damage. Final report for Cooperative Agreement DAMD17-94-V-4009, NTIS AD-A366 573.

LANGENBERG, J.P., BENSCHOP, H.P. AND VAN DER SCHANS, G.P. (2000) Inhalation and percutaneous toxicokinetics of sulfur mustard and its adducts in hairless guinea pigs and marmosets. Efficacy of TSPs and nasal scavengers. Proposal for Cooperative Agreement.

LIN, R.C., SHAHIDI, S., KELLY, T.J., LUMENG, C. AND LUMENG, L. (1993) Measurement of hemoglobin-acetaldehyde adduct in alcoholic patients. Alcohol Clin. Exp. Res. **17**, 669-674.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. AND RANDALL, R.J. (1951) Protein measurement with the Folin-Phenol reagent. J. Biol. Chem. **193**, 265-275.

LYNCH, A.M., MURRAY, S., ZHAO, K., GOODERHAM, N.J., BOOBIS, A.R. AND DAVIES, D.S. (1993) Molecular dosimetry of the food-borne carcinogen MeIQX using adducts of serum albumin. Carcinogenesis **14**, 191-194.

MOL, M.A.E. (1992) Molecular and cellular mechanisms of sulfur-mustard-induced lesions of human skin. Ph.D. Thesis, State University of Leiden, The Netherlands.

MOL, M.A.E. (2000) Implications of protein alkylation and proteolysis on vesication caused by sulfur mustard. Final Report for Cooperative Agreement DAMD17-97-2-7018 (in preparation).

NOORT, D., VERHEIJ, E.R., HULST, A.G., DE JONG, L.P.A. AND BENSCHOP, H.P. (1996a). Characterization of sulfur mustard induced structural modifications in human hemoglobin by LC-tandem mass spectrometry. Chem. Res. Toxicol. **9**, 781-787.

NOORT, D., PLATENBURG, D.H.J.M. AND BENSCHOP, H.P. (1996b) Solid phase synthesis of peptides containing a phosphoserine - sulfur mustard adduct. Bioorg. Med. Chem. Lett. **6**, 2007-2012.

NOORT, D., HULST, A.G., TRAP, H.C., DE JONG, L.P.A. AND BENSCHOP, H.P. (1997) Synthesis and mass spectrometric identification of the major amino acid adducts formed between sulfur mustard and hemoglobin in human blood. Arch. Toxicol. **71**, 171-178.

NOORT, D., HULST, A.G., DE JONG, L.P.A., AND BENSCHOP, H.P. (1999) Alkylation of human serum albumin by sulfur mustard *in vitro* and *in vivo*: mass spectrometric analysis of a cysteine adduct as a sensitive biomarker of exposure. Chem. Res. Toxicol. **12**, 715-721.

OTT, D.G., REISFELD, M.J. AND WHALEY, T.W. (1986) Synthesis and applications of isotopically labeled compounds. Proceedings of the Second International Symposium, Kansas City, MO (Muccino, R.R., Ed.). Elsevier, Amsterdam, pp. 409-414.

PUMFORD, N.R., MYERS, T.G., DAVILLA, J.C., HIGHET, R.J. AND POHL, L.R. (1993) Immunochemical detection of liver protein adducts of the nonsteroidal antiinflammatory drug diclofenac. Chem. Res. Toxicol. **6**, 147-150.

Report of the specialists appointed by the Secretary-General to investigate allegations by the Islamic Republic of Iran concerning the use of chemical weapons. UN Document S/16, 433 of 26 March 1984.

SANTELLA, R.M., LIN, C.D. AND DHARMARAJA, N. (1986) Monoclonal antibodies to a benzo[a]pyrene diolepoxide modified protein. Carcinogenesis **7**, 441-444.

SHEABAR, F.Z., GROOPMAN, J.D., QIAN G.S. AND WOGAN, G.N. (1993) Quantitative analysis of aflatoxin-albumin adducts. Carcinogenesis, **14**, 1203-1208.

SKIPPER, P.L. AND TANNENBAUM, S.R. (1990) Protein adducts in the molecular dosimetry of chemical carcinogens. Carcinogenesis **11**, 507-518.

SKIPPER, P.L., OBIEDZINSKI, M.W., TANNENBAUM, S.R., MILLER, D.W., MITCHUM, R.K. AND KADLUBAR, F.F. (1985) Identification of the major serum albumin adduct formed by 4-aminobiphenyl *in vivo* in rats. Cancer Res. **45**, 5122-5127.

SUN, T.-T. AND GREEN, H. (1978) Keratin filaments of cultural human epidermal cells. J. Biol. Chem. **253**, 2053-2060.

TALBOT, B., DESNOYERS, S. AND CASTONGUAY, A. (1990) Immunoassays for proteins alkylated by nicotine-derived N-nitrosamines. Arch. Toxicol. **64**, 360-364.

UCHIDA, K., TOYOKUNI, S., NISHIKAWA, K., KAWAKISHI, S., ODA, H., HIAI, H. AND STADTMAN, E.R. (1994) Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. Biochemistry **33**, 12487-12494.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., TIMMERMAN, A.J. AND BAAN, R.A. (1993) Progress in the development of the immunochemical assay for the detection of DNA-damage as a tool for biological dosimetry. Proceedings of the meeting of NATO AC/243 Panel 8 RSG 23, April 1993, Bethesda, MD, USA.

VAN DER SCHANS, G.P., SCHEFFER, A.G., MARS-GROENENDIJK, R.H., FIDDER, A., BENSCHOP, H.P. AND BAAN, R.A. (1994a) Immunochemical detection of adducts of sulfur mustard to DNA of calf thymus and human white blood cells. Chem. Res. Toxicol. **7**, 408-413.

VAN DER SCHANS, G.P., TIMMERMAN, A.J., MARS-GROENENDIJK, R.H. AND BAAN, R.A. (1994b) Validation of the immunochemical assay for the detection of DNA-damage as a tool for biological dosimetry of radiation exposure. Proceedings of the meeting of NATO AC/243 Panel 8 RSG 23, May 1994, Munich, Germany.

VAN DER SCHANS, G.P., VAN DIJK-KNIJNENBURG, W.C.M. AND MARS-GROENENDIJK, R.H. (1999) Unpublished results of TNO Prins Maurits Laboratory.

WRAITH, M.J., WATSON, W.P., EADSFORTH, C.V., VAN SITTERT, N.J. AND WRIGHT, A.S. (1988) An immunoassay for monitoring human exposure to ethylene oxide. In: Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention. Proceedings of a symposium held in Espoo, Finland, 2-4 September 1987 (Bartsch, H., Hemminki, K. and O'Neill, I.K., Eds). IARC Scientific Publication No 89, Lyon, pp. 271-274.

# X BIBLIOGRAPHY OF PUBLICATIONS AND MEETING ABSTRACTS

NOORT, D. (1997) Mass spectrometric detection of sulfur mustard adducts to DNA and hemoglobin.
Abstract book "5th European Meeting on Mass Spectrometry in Occupational and Environmental Health" (9-11 June, Nijmegen, The Netherlands), pp 63-64.

NOORT, D., FIDDER, A., DE JONG, L.P.A., AND BENSCHOP, H.P. (1998) Diagnosis and dosimetry of exposure to sulfur mustard: development of standard operating procedures for hemoglobin adducts; exploratory research on albumin and keratin adducts.
Program Book Bioscience Review (31 May - 4 June, 1998, Hunt Valley, MD), pp 116-117.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., DE JONG, L.P.A., BRUIJNZEEL, P.L.B., AND BENSCHOP, H.P. (1998) Immunochemical detection of sulfur mustard-adducts with DNA and proteins; development of standard operating procedures for DNA adducts and exploratory research on adducts with proteins.
Program Book Bioscience Review (31 May - 4 June, 1998, Hunt Valley, MD), pp 144-145.

NOORT, D., HULST, A.G., DE JONG, L.P.A., AND BENSCHOP, H.P. (1999) Alkylation of human serum albumin by sulfur mustard *in vitro* and *in vivo*: mass spectrometric analysis of a cysteine adduct as a sensitive biomarker of exposure.
Chem. Res. Toxicol. **12**, 715-721.

FIDDER, A., NOORT, D., AND BENSCHOP, H.P. (1999) A convenient synthesis of [$^{14}$C]1,1'-thiobis(2-chloroethane), [$^{14}$C]sulfur mustard.
J. Lab. Comp. Radiopharm. **42**, 261-266.

NOORT, D. (1999) An improved method for diagnosis of exposure to sulphur mustard: mass spectrometric analysis of adducts to human serum albumin.
Abstract Book "NATO TG 004 Meeting", March 22-26, Brussels.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., DE JONG, L.P.A., BRUIJNZEEL, P.L.B., AND BENSCHOP, H.P. (1999) Immunochemical detection of sulfur mustard-adducts with DNA and proteins; exploratory research on adducts with proteins.
Abstract Book "NATO TG 004 Meeting", March 22-26, Brussels.

BENSCHOP, H.P., NOORT, D., AND BLACK, R.M. (1999) Methods for retrospective detection of exposure to toxic scheduled chemicals: an overview. UK/NL Non-Paper to the OPCW; 6 December 1999.

NOORT, D., AND BENSCHOP, H.P. (2000) Methods for retrospective detection of exposure to toxic scheduled chemicals: an overview.
Abstract book "CB-MTS III symposium", May 7-12, 2000, Spiez, Switzerland.

## XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

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Mr. E. Wils
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Dr. B.L.M. van Baar
Mr. C.J.M. van der Wulp
Mrs. H. van der Wiel